

## Noninvasive Assessment of Tumor Hypoxia with $^{99m}\text{Tc}$ Labeled Metronidazole

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**Purpose.** The assessment of tumor hypoxia by imaging modality prior to radiation therapy would provide a rational means of selecting patients for treatment with radiosensitizers or bioreductive drugs. This study aimed to develop a  $^{99m}\text{Tc}$ -labeled metronidazole (MN) using ethylenedicycysteine (EC) as a chelator and evaluate its potential use to image tumor hypoxia.

**Methods.** EC was conjugated to amino analogue of MN using Sulfo-N-hydroxysuccinimide and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide-HCl as coupling agents, the yield was 55%. Tissue distribution of  $^{99m}\text{Tc}$ -EC-MN was determined in breast tumor-bearing rats at 0.5, 2, and 4 hrs. Planar imaging and whole-body autoradiograms were performed. The data was compared to that using  $^{99m}\text{Tc}$ -EC (control), [ $^{18}\text{F}$ ]fluoromisonidazole (FMISO) and [ $^{131}\text{I}$ ]iodomisonidazole (IMISO).

**Results.** *In vivo* biodistribution of  $^{99m}\text{Tc}$ -EC-MN in breast tumor-bearing rats showed increased tumor-to-blood and tumor-to-muscle ratios as a function of time. Conversely, tumor-to-blood values showed time-dependent decrease with  $^{99m}\text{Tc}$ -EC in the same time period. Planar images and autoradiograms confirmed that the tumors could be visualized clearly with  $^{99m}\text{Tc}$ -EC-MN from 0.5 to 4 hrs. There was no significant difference of tumor-to-blood count ratios between  $^{99m}\text{Tc}$ -EC-MN and [ $^{131}\text{I}$ ]IMISO at 2 and 4 hrs postinjection. From 0.5 to 4 hrs, both  $^{99m}\text{Tc}$ -EC-MN and [ $^{131}\text{I}$ ]IMISO have higher tumor-to-muscle ratios compared to [ $^{18}\text{F}$ ]FMISO.

**Conclusions.** It is feasible to use  $^{99m}\text{Tc}$ -EC-MN to image tumor hypoxia.

**KEY WORDS:** metronidazole;  $^{99m}\text{Tc}$ ; tumor hypoxia; imaging; radiosensitizer.

### INTRODUCTION

Tumor cells are more sensitive to conventional radiation in the presence of oxygen than in its absence; even a small percentage of hypoxic cells within a tumor could limit the response to radiation (1–3). Hypoxic radioresistance has been demonstrated in many animal tumors but only in a few tumor types in humans (4–6). The occurrence of hypoxia in human tumors has, in most cases, been inferred from histology findings and from animal tumor studies. *In vivo* demonstration of hypoxia has required tissue measurements with oxygen electrodes, and the invasiveness of these techniques has limited their clinical application.

Misonidazole (MISO) is a hypoxic cell sensitizer, and labeling MISO with different radioisotopes (e.g.,  $^{18}\text{F}$ ,  $^{123}\text{I}$ ,  $^{99m}\text{Tc}$ ) may be useful for differentiating a hypoxic but metabolically active tumor from a well-oxygenated active tumor by positron emission tomography (PET) or planar scintigraphy. Moreover, the assessment of tumor hypoxia with labeled MISO prior to radiation therapy would provide a rational means of selecting patients for treatment with radiosensitizing or bioreductive drugs (e.g. mitomycin C). Such selection of patients would permit more accurate treatment because use of these modalities could be limited to patients with hypoxic tumors. It is also possible to select proper modalities of radiotherapy (neutron vs. photon) by correlating labeled MISO results with tumor response.

[ $^{18}\text{F}$ ]Fluoromisonidazole (FMISO) has been used with PET to evaluate tumor hypoxia. Recent studies have shown that PET, with its ability to monitor cell oxygen content through [ $^{18}\text{F}$ ]FMISO, has a high potential to predict tumor response to radiation (7–12). PET gives a higher resolution without collimation, however, the cost of using PET isotopes in a clinical setting is prohibitive. Although labeling MISO with iodine was the choice, high uptake in thyroid tissue was observed (13). Therefore, it is desirable to develop compounds for planar scintigraphy that the isotope is less expensive and easily available in most major medical facilities.

*In vitro* studies using mammalian cells, metronidazole (MN) was shown to sensitize only anoxic cells in a dose dependent manner, a maximum enhancement ratio of 1.9 being obtained (2.5 for MISO). However, *in vivo* studies showed that this sensitizer plus radiation group had survival equivalent to that of patients given standard fractionated high-dose radiation. The difference in sensitization effect may be due to electron affinity, lipophilic character (to reduce systemic toxicity and not to lose sensitization activity) and glutathione depletion characteristics (14).

Due to favorable physical characteristics as well as extremely low price,  $^{99m}\text{Tc}$  have been preferred to label radiopharmaceuticals. Several nitroimidazole analogues have been labeled with  $^{99m}\text{Tc}$  using nitrogen and sulfur chelates (15–18). Bis-aminoethanethiol tetradentate ligands, also called diamino-dithiol compounds, are known to form very stable Tc(V)O-complexes on the basis of efficient binding of the oxotechnetium group to two thiol sulphur and two amine nitrogen atoms (19,20).  $^{99m}\text{Tc}$ -L,L-ethylenedicycysteine ( $^{99m}\text{Tc}$ -EC) is a recent and successful example of  $\text{N}_2\text{S}_2$  chelates. EC can be labeled with  $^{99m}\text{Tc}$  easily and efficiently with high radiochemical purity and stability and is excreted through kidney by active tubular transport (20–22).

Although radiosensitizing effect using MN was not impressive, MN has been shown to sensitize hypoxic tumors. Thus, in this paper, we present the synthesis of  $^{99m}\text{Tc}$ -labeled metronidazole using EC as a chelator and evaluate its potential use as a tumor hypoxic imaging agent and results were compared to those  $^{99m}\text{Tc}$ -EC (standard), [ $^{18}\text{F}$ ]FMISO and [ $^{131}\text{I}$ ]IMISO.

### MATERIALS AND METHODS

The nuclear magnetic resonance (NMR) and mass spectral analysis were conducted at the University of Texas Health Science Center (Houston, TX). NMR spectra were recorded on

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a GE GN-500 Spectrometer. The mass data were obtained by fast atom bombardment on a Kratos MS 50 instrument (England). Sulfo-N-hydroxysuccinimide (Sulfo-NHS) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide-HCl (EDC) were purchased from Pierce Chemical Co (Radford, IL). All other chemicals were purchased from Aldrich Chemical Co (Milwaukee, WI). Silica gel coated thin-layer chromatography (TLC) plate was purchased from Whatman (Clifton, NJ).  $^{99m}\text{Tc}$ -pertechnetate was obtained from a commercial  $^{99}\text{Mo}/^{99m}\text{Tc}$  generator (Ultratechnekow FM™, Mallinckrodt Diagnostica, Holland).

#### Synthesis of L,L-Ethylenedicysteine (EC)

EC was prepared in a two-step synthesis according to the previously described methods (23,24). The precursor, L-thiazolidine-4-carboxylic acid, was synthesized (m.p. 195°, reported 196–197°). EC was then prepared (m.p. 237°, reported 251–253°C). The structure was confirmed by  $^1\text{H-NMR}$  and mass spectroscopy (FAB-MS)  $m/z$  268 ( $\text{M}^+$ , 100).

#### Synthesis of 2-(2-Methyl-5-Nitro-1H-Imidazolyl)Ethylamine (Amino Analogue of Metronidazole, MN-NH<sub>2</sub>)

Amino analogue of metronidazole was synthesized according to the previously described methods (25). Briefly, metronidazole was converted to a mesylated analogue (m.p. 149–150°C, reported 153–154°C, TLC:ethyl acetate,  $R_f$  = 0.45), yielded 75%. Mesylated metronidazole was then reacted with sodium azide to afford azido analogue (TLC:ethyl acetate,  $R_f$  = 0.52), yielded 80%. The azido analogue was reduced by triphenyl phosphine and yielded (60%) the desired amino analogue (m.p. 190–192°C, reported 194–195°C, TLC:ethyl acetate,  $R_f$  = 0.15). Ninhydrin (2% in methanol) spray indicated the positivity of amino group of MN-NH<sub>2</sub>. The structure was confirmed by  $^1\text{H-NMR}$  and mass spectroscopy (FAB-MS)  $m/z$  171 ( $\text{M}^+\text{H}$ , 100).

#### Synthesis of Ethylenedicysteine-Metronidazole (EC-MN)

Sodium hydroxide (2N, 0.2 ml) was added to a stirred solution of EC (134 mg, 0.50 mmol) in water (5 ml). To this colorless solution, sulfo-NHS (217 mg, 1.0 mmol) and EDC (192 mg, 1.0 mmol) were added. MN-NH<sub>2</sub> dihydrochloride salt (340 mg, 2.0 mmol) was then added. The mixture was stirred at room temperature for 24 hours. The mixture was dialyzed for 48 hrs using Spectra/POR molecular porous membrane with cut-off at 500 (Spectrum Medical Industries Inc., Houston, TX). After dialysis, the product was frozen dried using lyophilizer (Labconco, Kansas City, MO). The product weighed 315 mg (yield 55%).  $^1\text{H-NMR}$  ( $\text{D}_2\text{O}$ )  $\delta$  2.93 (s, 6H, nitroimidazole- $\text{CH}_3$ ), 2.60–2.95 (m, 4H and  $-\text{CH}_2-\text{SH}$  of EC), 3.30–3.66 (m, 8H, ethylenediamine of EC and nitromidazole- $\text{CH}_2-\text{CH}_2-\text{NH}_2$ ), 3.70–3.99 (t, 2H, NH- $\text{CH}-\text{CO}$  of EC), 5.05 (t, 4H, metronidazole- $\text{CH}_2-\text{CH}_2-\text{NH}_2$ ) (s, 2H, nitroimidazole  $\text{C}=\text{CH}$ ). FAB MS  $m/z$  572 ( $\text{M}^+$ , 20). The synthetic scheme of EC-MN is shown in Fig. 1.

#### Radiolabeling of EC-MN and EC with $^{99m}\text{Tc}$

Radiosynthesis of  $^{99m}\text{Tc}$ -EC-MN was achieved by adding required amount of  $^{99m}\text{Tc}$ -pertechnetate into home-made kit

containing the lyophilized residue of EC-MN (3 mg),  $\text{SnCl}_2$  (100  $\mu\text{g}$ ),  $\text{Na}_2\text{HPO}_4$  (13.5 mg), ascorbic acid (0.5 mg) and NaEDTA (0.5 mg). Final pH of preparation was 7.4.  $^{99m}\text{Tc}$ -EC was also obtained by using home-made kit containing the lyophilized residue of EC (3 mg),  $\text{SnCl}_2$  (100  $\mu\text{g}$ ),  $\text{Na}_2\text{HPO}_4$  (13.5 mg), ascorbic acid (0.5 mg) and NaEDTA (0.5 mg) at pH 10. Final pH of preparation was then adjusted to 7.4. Radiochemical purity was determined by TLC (ITLC SG, Gelman Sciences, Ann Arbor, MI) eluted with acetone (system A) and ammonium acetate (1M in water):methanol (4:1) (system B), respectively. From radio-TLC (Bioscan, Washington, DC) analysis, the radiochemical purity was >95% for both radiotracers.

#### Synthesis of [ $^{18}\text{F}$ ]FMISO and [ $^{131}\text{I}$ ]IMISO

[ $^{18}\text{F}$ ]fluoride was produced by the cyclotron using proton irradiation of enriched  $^{18}\text{O}$ -water in a small-volume silver target. The tosyl MISO (26) (20 mg) was dissolved in acetonitrile (1.5 ml), added to the kryptofix-fluoride complex. After heating, hydrolysis and column purification, a yield of 25–40% (decay corrected) of pure product was isolated with the end of bombardment (EOB) at 60 min. HPLC was performed on a C-18 ODS-120T column, 4.6  $\times$  25 mm (Waters Corp., Milford, Mass), with water/acetonitrile, (80/20), using a flow rate of 1 ml/min. The no-carrier-added product corresponded to the retention time (6.12 min) of the unlabeled FMISO under similar conditions. The radiochemical purity was greater than 99%. Under the UV detector (310 nm), there were no other impurities. The specific activity of [ $^{18}\text{F}$ ]FMISO determined was 1 Ci/ $\mu\text{mol}$  based upon UV and radioactivity detection of a sample of known mass and radioactivity.

[ $^{131}\text{I}$ ]IMISO was prepared using the same precursor (26), briefly, 5 mg of tosyl MISO was dissolved in acetonitrile (1 ml), and  $\text{Na}^{131}\text{I}$  (1 mCi in 0.1 ml 1N NaOH) (Dupont New England Nuclear, Boston, MA) was added. After heating and purification, the product (60–70% yield) was obtained. Radio-TLC indicated the  $R_f$  values of 0.01 for the final product using chloroform methanol (7:3) as an eluant.

#### Stability Assay of $^{99m}\text{Tc}$ -EC-MN

Stability of labeled  $^{99m}\text{Tc}$ -EC-MN was tested in serum samples. Briefly, 740 KBq of 1 mg  $^{99m}\text{Tc}$ -EC-MN was incubated in dog serum (200  $\mu\text{l}$ ) at 37°C for 4 hours. The serum samples was diluted with 50% methanol in water and radio-TLC repeated at 0.5, 2 and 4 hours as described above.

#### Tissue Distribution Studies

The animal experiments were approved by The University of Texas M. D. Anderson Institutional Animal Care and Use Committee (IACUC). Female Fischer 344 rats (150  $\pm$  25 g) (Harlan Sprague-Dawley, Indianapolis, IN) were inoculated subcutaneously with 0.1 ml of mammary tumor cells from the 13762 tumor cell line suspension ( $10^6$  cells/rat, a tumor cell line specific to Fischer rats) into the hind legs using 25-gauge needles. Studies performed 14 to 17 days after implantation when tumors reached approximately 1 cm diameter. Rats were anesthetized with ketamine (10–15 mg/rat, intraperitoneally) before each procedure.

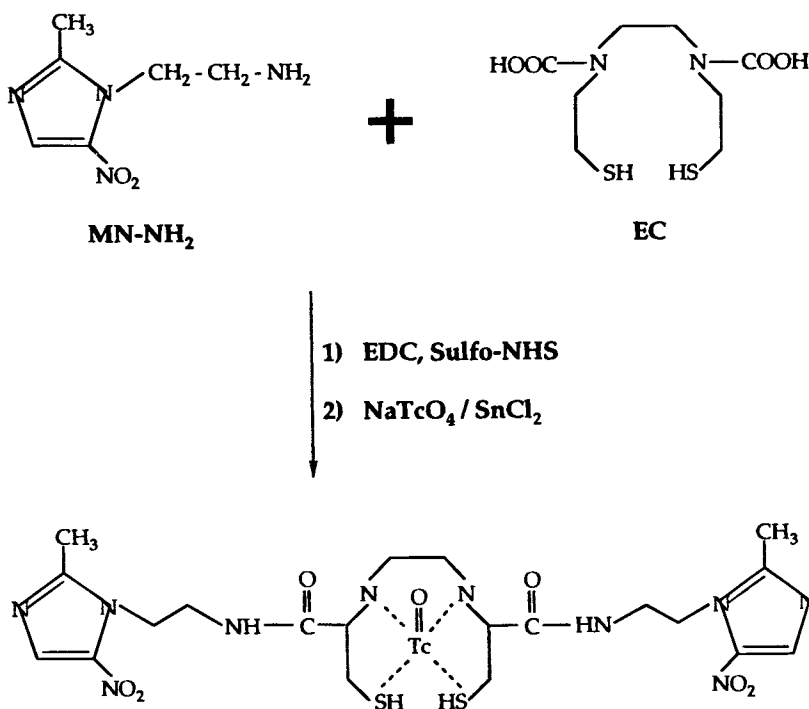


Fig. 1. Radiosynthesis of  $^{99m}\text{Tc}$ -EC-metronidazole (EC-MN).

In tissue distribution studies, each animal was injected intravenously with 370–550 KBq of  $^{99m}\text{Tc}$ -EC-MN or  $^{99m}\text{Tc}$ -EC ( $n = 3/\text{time point}$ ). The injected mass of  $^{99m}\text{Tc}$ -EC-MN was 10  $\mu\text{g}$  per rat. At 0.5, 2 and 4 hrs following administration of the radiotracers, the rats were sacrificed and the selected tissues were excised, weighed and counted for radioactivity. The biodistribution of tracer in each sample was calculated as percentage of the injected dose per gram of tissue wet weight (%ID/g). Tumor/nontarget tissue count density ratios were calculated from the corresponding %ID/g values. The data was compared to [ $^{18}\text{F}$ ]FMISO and [ $^{131}\text{I}$ ]IMISO using the same animal model. Student *t*-test was used to assess the significance of differences between groups.

#### Scintigraphic Imaging and Autoradiography Studies

Scintigraphic images, using a gamma camera (Siemens Medical Systems, Inc., Hoffman Estates, IL) equipped with low-energy, parallel-hole collimator, were obtained 0.5, 2 and 4 hrs after i.v. injection of 18.5 MBq of each radiotracer.

Whole-body autoradiogram was obtained by a quantitative image analyzer (Cyclone Storage Phosphor System, Packard, Meridian, CT). Following i.v. injection of 37 MBq of  $^{99m}\text{Tc}$ -EC-MN, animal was killed at 1 h and the body was fixed in carboxymethyl cellulose (4%) as previously described (12,13). The frozen body was mounted onto a cryostat (LKB 2250 cryomicrotome) and cut into 100  $\mu\text{m}$  coronal sections. Each section was thawed and mounted on a slide. The slide was then placed in contact with multipurpose phosphor storage screen (MP, 7001480) and exposed for 15 hrs.

#### Polarographic Oxygen Microelectrode $\text{pO}_2$ Measurements

To confirm tumor hypoxia, intratumoral  $\text{pO}_2$  measurements were performed using the Eppendorf computerized histographic system. Twenty to twenty-five  $\text{pO}_2$  measurements along each of two to three linear tracks were performed at 0.4 mm intervals on each tumor (40–75 measurements total). Tumor  $\text{pO}_2$  measurements were made on three tumor-bearing rats. Using an on-line computer system, the  $\text{pO}_2$  measurements of each track were expressed as absolute values relative to the location of the measuring point along the track, and as the relative frequencies within a  $\text{pO}_2$  histogram between 0 and 100 mmHg with a class width of 2.5 mm.

Table 1. Biodistribution of  $^{99m}\text{Tc}$ -EC-Metronidazole Conjugate in Breast Tumor Bearing Rats<sup>a</sup>

	30 min	2 hr	4 hr
Blood	1.46 ± 0.73	1.19 ± 0.34	0.76 ± 0.14
Lung	0.79 ± 0.39	0.73 ± 0.02	0.52 ± 0.07
Liver	0.83 ± 0.36	0.91 ± 0.11	0.87 ± 0.09
Spleen	0.37 ± 0.17	0.41 ± 0.04	0.37 ± 0.07
Kidney	4.30 ± 1.07	5.84 ± 0.43	6.39 ± 0.48
Muscle	0.08 ± 0.03	0.09 ± 0.01	0.07 ± 0.01
Intestine	0.27 ± 0.12	0.39 ± 0.24	0.22 ± 0.05
Thyroid	0.51 ± 0.16	0.51 ± 0.09	0.41 ± 0.02
Tumor	0.34 ± 0.13	0.49 ± 0.02	0.50 ± 0.09

<sup>a</sup> Each rat received  $^{99m}\text{Tc}$ -EC-metronidazole (10  $\mu\text{Ci}$ , iv). Each value is percent of injected dose per gram weight ( $n = 3$ )/time interval. Each data represents mean of three measurements with standard deviation.

**Table 2.** Biodistribution of  $^{99m}\text{Tc}$ -EC in Breast Tumor-Bearing Rats<sup>a</sup>

	% of injected $^{99m}\text{Tc}$ -EC dose per organ or tissue		
	30 min	2 hr	4 hr
Blood	0.44 ± 0.03	0.21 ± 0.01	0.15 ± 0.01
Lung	0.27 ± 0.02	0.14 ± 0.01	0.12 ± 0.01
Liver	0.51 ± 0.06	0.29 ± 0.07	0.23 ± 0.02
Stomach	0.14 ± 0.06	0.04 ± 0.03	0.04 ± 0.01
Kidney	7.91 ± 0.90	9.12 ± 0.05	7.83 ± 1.02
Muscle	0.06 ± 0.01	0.03 ± 0.01	0.02 ± 0.01
Intestine	0.17 ± 0.03	0.40 ± 0.09	0.10 ± 0.01
Thyroid	0.22 ± 0.04	0.11 ± 0.00	0.08 ± 0.01
Tumor	0.34 ± 0.16	0.12 ± 0.00	0.10 ± 0.01

<sup>a</sup> Values shown represent the mean ± standard deviation of data from 3 animals.

## RESULTS

### Radiosynthesis and Stability of $^{99m}\text{Tc}$ -EC-MN

Radiosynthesis of EC-MN with  $^{99m}\text{Tc}$  was achieved with high (>95%) radiochemical purity. Radiochemical yield was 100%.  $^{99m}\text{Tc}$ -EC-MN was found to be stable at 0.5, 2 and 4 hrs in dog serum samples. There was no degradation products observed. Radiofluorination and radioiodination of MISO were

achieved easily using the same precursor. In both labeled MISO analogues, the radiochemical purity was greater than 99%.

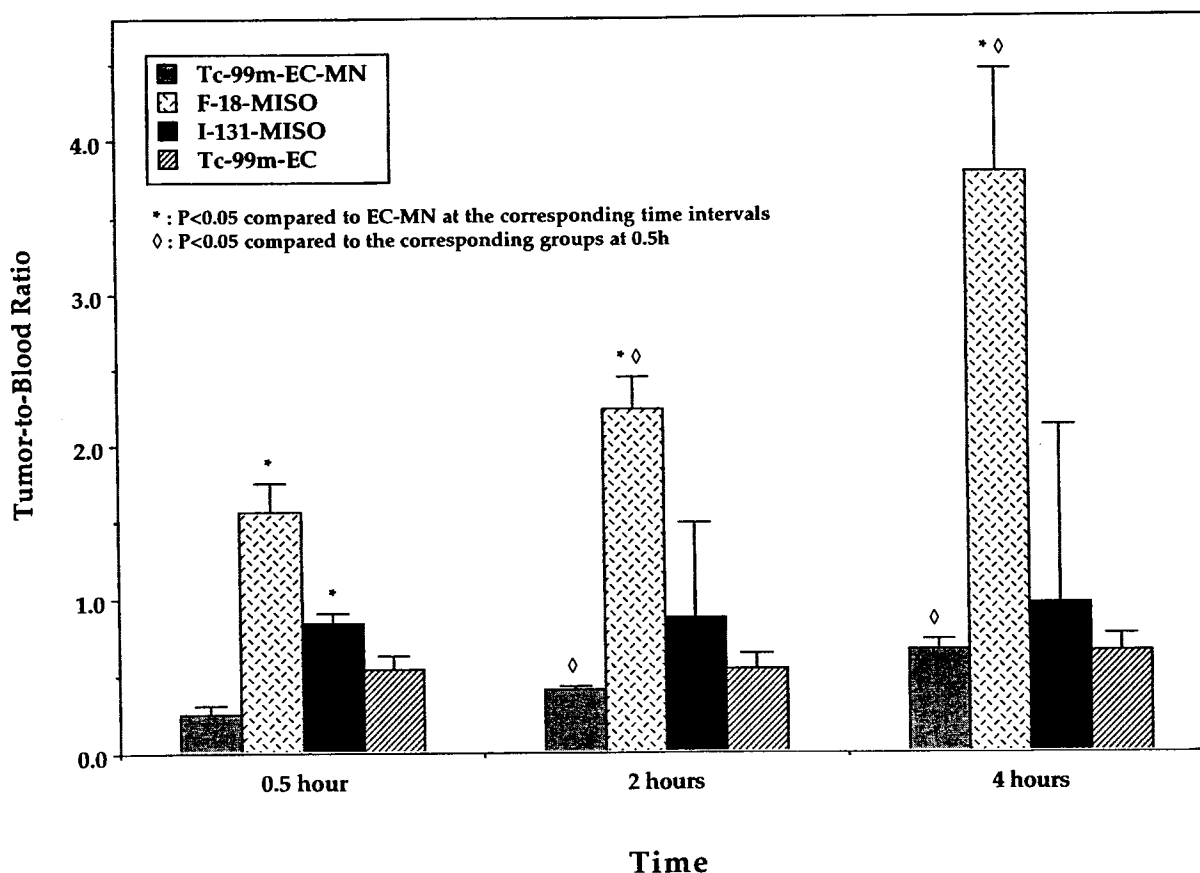
### In Vivo Tissue Distribution Studies

The tissue distribution of  $^{99m}\text{Tc}$ -EC-MN and  $^{99m}\text{Tc}$ -EC in the tumor-bearing rats is shown in Tables 1 and 2. Due to high affinity for ionic  $^{99m}\text{Tc}$ , there was no significant and consistent thyroid uptake, suggesting the in vivo stability of  $^{99m}\text{Tc}$ -EC-MN (Table 1).

Biodistribution studies showed that tumor/blood and tumor/muscle count density ratios at 0.5-4 hr gradually increased for  $^{99m}\text{Tc}$ -EC-MN, [ $^{18}\text{F}$ ]FMISO and [ $^{131}\text{I}$ ]IMISO, whereas these values did not alter for  $^{99m}\text{Tc}$ -EC in the same time period (Figs. 2 and 3). [ $^{18}\text{F}$ ]FMISO showed the highest tumor-to-blood uptake ratio than those with [ $^{131}\text{I}$ ]IMISO and  $^{99m}\text{Tc}$ -EC-MN at 30 min, 2 and 4 hrs post-injection. Tumor/blood and tumor/muscle ratios for  $^{99m}\text{Tc}$ -EC-MN and [ $^{131}\text{I}$ ]IMISO at 2 and 4 hrs postinjection were not significantly different ( $p < 0.05$ ).

### Scintigraphic Imaging and Autoradiographic Studies

Scintigraphic images obtained at different time points showed visualization of tumor in  $^{99m}\text{Tc}$ -EC-MN group. Contrary, there was no apparent tumor uptake in  $^{99m}\text{Tc}$ -EC injected group (Fig. 4). Autoradiograms performed at 1 hr after injection of  $^{99m}\text{Tc}$ -EC-MN clearly demonstrated tumor activity (Fig. 5).



**Fig. 2.** Time-dependent variation of tumor/blood uptake ratios (%ID/g wet weight) with  $^{99m}\text{Tc}$ -EC-MN, [ $^{18}\text{F}$ ]FMISO, [ $^{131}\text{I}$ ]IMISO versus  $^{99m}\text{Tc}$ -EC ( $n = 3$ /time point).

### Polarographic Oxygen Microelectrode $pO_2$ Measurements

Intratumoral  $pO_2$  measurements of tumors indicated the tumor oxygen tension ranged  $4.6 \pm 1.4$  mmHg as compared to normal muscle of  $35 \pm 10$  mmHg. The data indicate that the tumors are hypoxic.

### DISCUSSION

The development of new tumor hypoxia agents is clinically desirable for detecting primary and metastatic lesions as well as predicting radioresponsiveness and time to recurrence. None of the contemporary imaging modalities accurately measures hypoxia since the diagnosis of tumor hypoxia requires pathologic examination. It is often difficult to predict the outcome of a therapy for hypoxic tumor without knowing at least the baseline of hypoxia in each tumor treated. Although the Eppendorf polarographic oxygen microelectrode can measure the oxygen tension in a tumor this technique is invasive and needs a skillful operator. Additionally, this technique can only be used on accessible tumors (e.g. head and neck, cervical) and multiple readings are needed. Therefore, an accurate and easy method of measuring tumor hypoxia will be useful for patient selection. However, tumor to normal tissue uptake ratios varies depend upon the radiopharmaceuticals used. Therefore, it would be rational to correlate tumor to normal tissue uptake ratio with the gold standard Eppendorf electrode measures of hypoxia when new radiopharmaceuticals introduced to clinical practice.

$[^{18}F]FMISO$  has been used to diagnose head and neck tumors, myocardial infarction, inflammation, and brain ischemia (27–30). Tumor to normal tissue uptake ratio was used as a baseline to assess tumor hypoxia (29). Although tumor hypoxia using  $[^{18}F]FMISO$  was clearly demonstrated, introducing new imaging agents into clinical practice depends on to some other factors such as easy availability and cost.

Due to better imaging characteristics and lower price attempts are made to replace the  $^{123}I$ ,  $^{131}I$ ,  $^{67}Ga$  and  $^{111}In$  labeled compounds with corresponding  $^{99m}Tc$  labeled compounds when possible. Verbruggen et al. reported that EC can be labeled with  $^{99m}Tc$  very easily and efficiently at room temperature with high radiochemical purity and the preparation remains stable for at least 8 hours (20). Because of reported labeling capacity and rapid renal clearance, EC was selected to synthesize a new  $^{99m}Tc$ -labeled metronidazole. EC-MN was prepared using a relatively simple and fast chemistry. A labeling kit was also developed to make its use in clinical practice easier. Radio-TLC results with  $^{99m}Tc$ -EC-MN kit confirm the high radiochemical purity and stability.

In tissue distribution studies, although no significance difference of tumor-to-tissue uptake between  $^{99m}Tc$ -EC-MN and  $^{99m}Tc$ -EC groups was observed, there was a significantly increased tumor-to-tissue uptake ratio as a function of time in the  $^{99m}Tc$ -EC-MN group. When compared with  $[^{18}F]FMISO$  and  $[^{131}I]IMISO$ , the tumor-to-tissue uptake ratios for  $^{99m}Tc$ -EC-MN is similar to those with  $[^{131}I]IMISO$ .

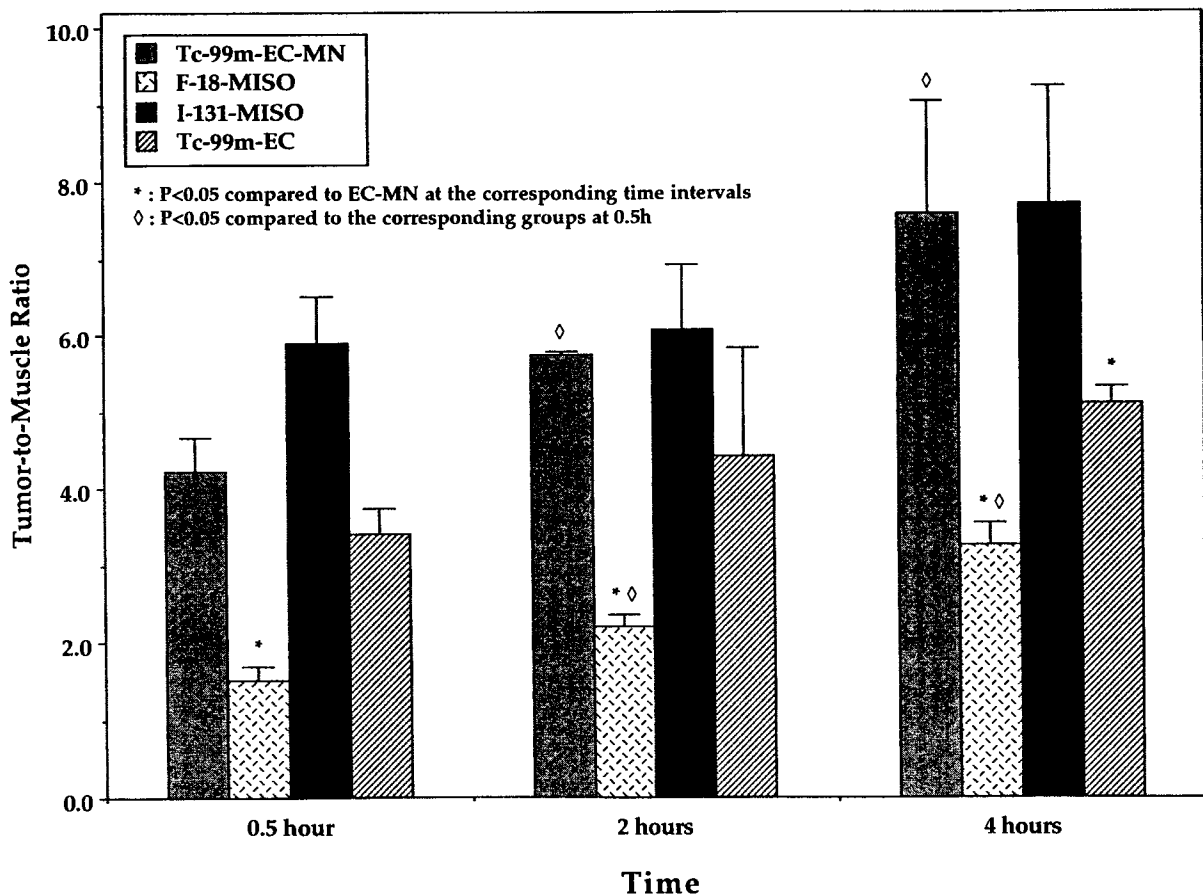
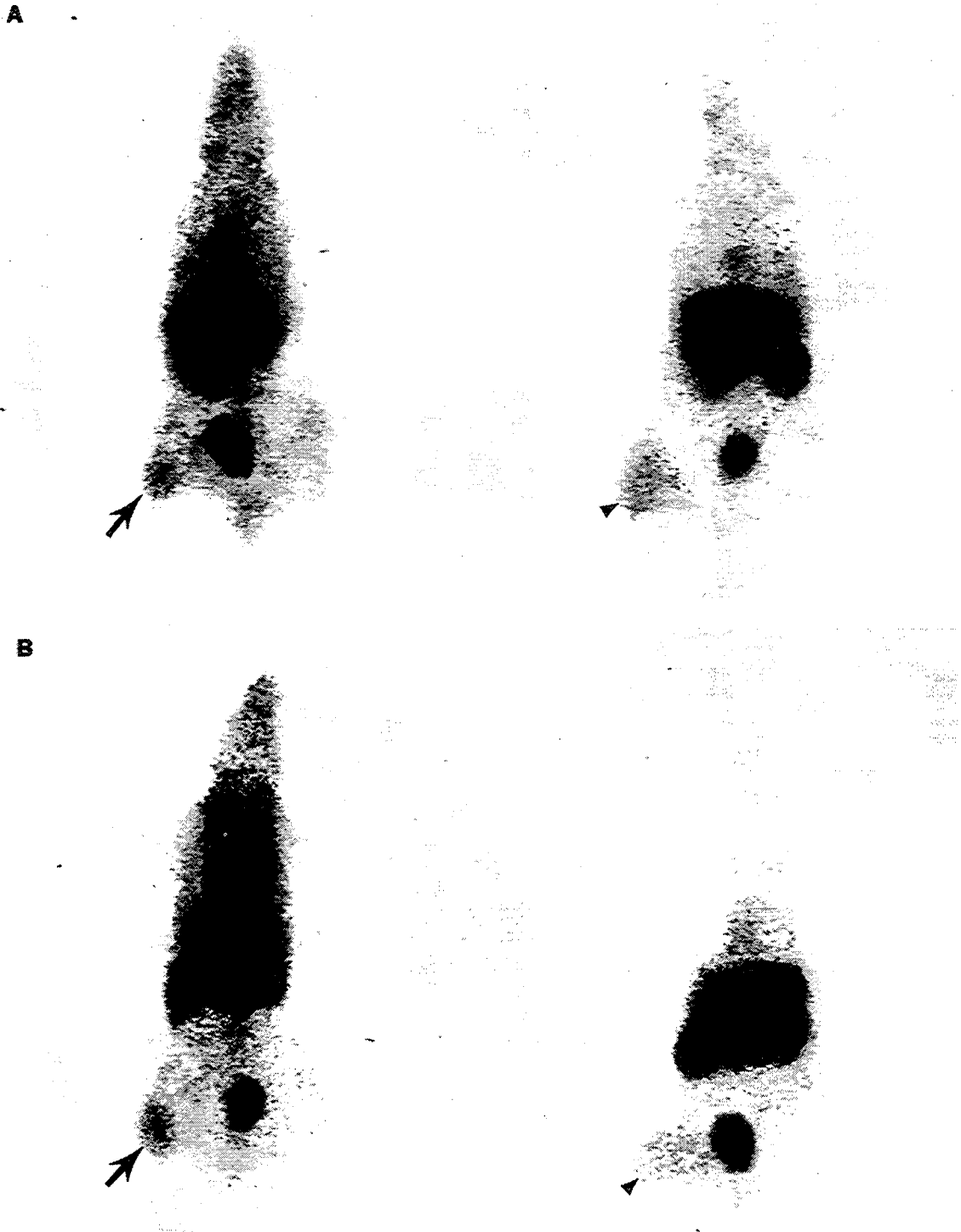


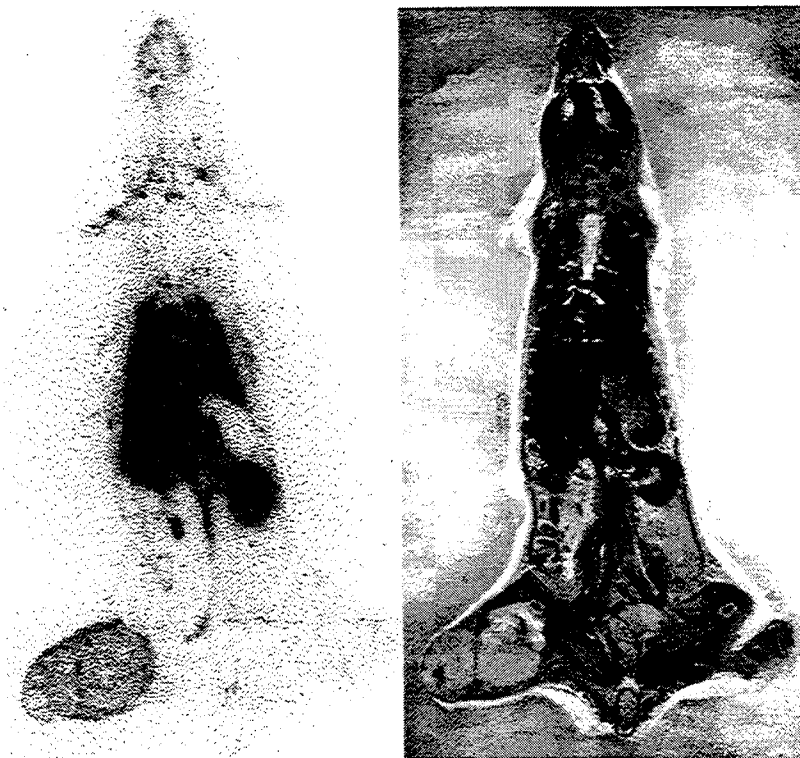
Fig. 3. Time-dependent variation of tumor/muscle uptake ratios (%ID/g wet weight) with  $^{99m}Tc$ -EC-MN,  $[^{18}F]FMISO$ ,  $[^{131}I]IMISO$  versus  $^{99m}Tc$ -EC (n = 3/time point).



**Fig. 4.** Selected gamma scintigraphy images (ventral view) of a rat with a subcutaneous breast tumor in the right hind leg at 1 (A) and 4 (B) hrs following intravenous administration of  $^{99m}\text{Tc}$ -EC-MN (left) and  $^{99m}\text{Tc}$ -EC (right).  $^{99m}\text{Tc}$ -EC-MN uptake was evident in the tumor (arrows) up to 4 hr. There was very minimal radiotracer uptake (arrowheads) in the tumor area with  $^{99m}\text{Tc}$ -EC.

Thyroid tissue uptake was not altered after  $^{99m}\text{Tc}$ -EC-MN, whereas thyroid uptake increased with [ $^{131}\text{I}$ ]IMISO. The findings suggest that  $^{99m}\text{Tc}$ -EC-MN is more metabolically stable than [ $^{131}\text{I}$ ]IMISO. Tumor oxygen tension was determined to be 3.2 to 6.0 mm-Hg, whereas normal muscle tissue had 30 to

40 mmHg. Although another factor such as anemia that may influence the level of tumor hypoxia, there was no attempt in identifying this factor. Autoradiograms of  $^{99m}\text{Tc}$ -EC-MN demonstrated the feasibility to assess tumor hypoxia. The findings support that further studies on determining normal tissue dosimetry



**Fig. 5.** Whole-body autoradiogram (coronal section) obtained 30 min after intravenous injection of  $^{99m}\text{Tc}$ -EC-MN clearly demonstrated tumor activity. A corresponding sectional image is on the right.

etry, measuring sensitizer enhancement ratio (SER) and identifying whether  $^{99m}\text{Tc}$ -EC-MN can provide a rational means of selecting patients for treatment with radiosensitizing (e.g. SR-2508, Ro-03-8799) or bioreductive agents.

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#### REFERENCES

1. E. J. Hall. The oxygen effect and reoxygenation. In E. J. Hall (ed.) *Radiobiology for the radiobiologist*, 3rd edition. J. B. Lippincott Co., Philadelphia, PA, 1988, pp. 137–160.
2. R. S. Bush, R. D. T. Jenkins, W. E. C. Allt, F. A. Beale, H. Bena, A. J. Dembo, and J. F. Pringle. Definitive evidence for hypoxic cells influencing cure in cancer therapy. *Br. J. Cancer* **37** (Suppl. III):302–306 (1978).
3. L. H. Gray, A. D. Conger, M. Elbert, S. Morsney, and O.C.A. Scold. The concentration of oxygen dissolved in tissues at the time of irradiation as a factor in radiotherapy. *Br. J. Radiol.* **26**:638–648 (1953).
4. S. Dische. A review of hypoxic-cell radiosensitization. *Int. J. Radiat. Oncol. Biol. Phys.* **20**:147–152 (1991).
5. R. A. Gatenby, H. B. Kessler, J. S. Rosenblum, L. R. Coia, P. J. Moldofsky, W. H. Hartz, and G. J. Broder. Oxygen distribution in squamous cell carcinoma metastases and its relationship to outcome of radiation therapy. *Int. J. Radiat. Oncol. Biol. Phys.* **14**:831–838 (1988).
6. M. Nordmark, M. Overgaard, and J. Overgaard. Pretreatment oxygenation predicts radiation response in advanced squamous cell carcinoma of the head and neck. *Radiother. Oncol.* **41**:31–39 (1996).
7. W-J Koh, J. S. Rasey, M. L. Evans, J. R. Grierson, T. K. Lewellen, M. M. Graham, K. A. Krohn, and T. W. Griffin. Imaging of hypoxia in human tumors with  $^{18}\text{F}$  fluoromisonidazole. *Int. J. Radiat. Oncol. Biol. Phys.* **22**:199–212 (1992).
8. P. E. T. Valk, C. A. Mathis, M. D. Prados, J. C. Gilbert, and T. F. Budinger. Hypoxia in human gliomas: Demonstration by PET with  $^{18}\text{F}$  fluoromisonidazole. *J. Nucl. Med.* **33**:2133–2137 (1992).
9. G. V. Martin, J. H. Caldwell, J. S. Rasey, Z. Grunbaum, M. Cerqueira, and K. A. Krohn. Enhanced binding of the hypoxic cell marker  $^{18}\text{F}$  fluoromisonidazole in ischemic myocardium. *J. Nucl. Med.* **30**:194–201 (1989).
10. J. S. Rasey, W. J. Koh, J. R. Grierson, Z. Grunbaum, and K. A. Krohn. Radiolabeled fluoromisonidazole as an imaging agent for tumor hypoxia. *Int. J. Radiat. Oncol. Biol. Phys.* **17**:985–991 (1989).
11. J. S. Rasey, N. J. Nelson, L. Chin, M. L. Evans, and Z. Grunbaum. Characterization of the binding of labeled fluoromisonidazole in cells in vitro. *Radiat. Res.* **122**:301–308 (1990).
12. D. J. Yang, S. Wallace, A. Cherif, C. Li, M. B. Gretzer, E. E. Kim, and D. A. Podoloff. Development of F-18-labeled fluoroe-rythronitroimidazole as a PET agent for imaging tumor hypoxia. *Radiology* **194**:795–800 (1995).
13. A. Cherif, S. Wallace, D. J. Yang, R. Newman, V. Wilson, A. Nornoo, T. Inoue, C. Kim, L-R Kuang, E. E. Kim, and D. A. Podoloff. Development of new markers for hypoxic cells:  $^{131}\text{I}$ iodomisonidazole and  $^{131}\text{I}$ iodoerythronitroimidazole. *J. Drug Targeting* **4**:31–39 (1996).
14. B. A. Teicher and E. A. Sotomayor. Chemical radiation sensitizers and protectors. In W. O. Foye (ed.), *Cancer Chemotherapeutic Agents*, American Chemical Society, Washington, D. C., 1995, pp. 501–527.

15. G. Johnson, K. N. Nguyen, Z. Lui, P. Gao, B. Edwards, C. M. Archer, A. C. King, T. L. North, R. D. Okada, and W. K. Warren. HL91 Technetium-99m: Kinetics of a new hypoxia avid imaging agent in normal and ischemic myocardium as assessed by gamma camera images. *J. Am. Coll. Cardiol.* 407A (1996).
16. K. Fukuchi, H. Kusuoka, K. Yutani, S. Hasegawa, and T. Nishimura. Assessment of reperfused myocardium using new hypoxia avid imaging agent Tc-99m HL91. *J. Nucl. Med.* 37:94 (Abstract) (1996).
17. T. Melo, J. Duncan, J. R. Ballinger, and A. M. Rauth. BMS 194796, a second generation Tc-99m labelled 2-nitroimidazole for imaging hypoxia in tumors. *J. Nucl. Med.* 39:219 (Abstract) (1998).
18. X. Zhang, T. Melo, J. R. Ballinger, and A. M. Rauth. Evaluation of Tc-99m butyleneamino oxime (BnAO), a non-nitroaromatic agent for imaging hypoxia in tumors. *J. Nucl. Med.* 39:216 (Abstract) (1998).
19. A. Davison, A. G. Jones, C. Orvig, and M. Sohn. A new class of oxotechnetium(+5) chelate complexes containing a TcON<sub>2</sub>S<sub>2</sub> Core. *Inorg. Chem.* 20:1629–1632 (1981).
20. A. M. Verbruggen, D. L. Nosco, C. G. Van Nerom, G. M. Bormans, P. J. Adriaens, and M. J. De Roo. Tc-99m-L,L-ethylenedicycysteine: A renal imaging agent. I. Labelling and evaluation in animals. *J. Nucl. Med.* 33:551–557 (1992).
21. C. G. Van Nerom, G. M. Bormans, M. J. De Roo, and A. M. Verbruggen. First experience in healthy volunteers with Tc-99m-L,L-ethylenedicycysteine, a new renal imaging agent. *Eur. J. Nucl. Med.* 20:738–746 (1993).
22. M. J. Surma, J. Wiewiora, and J. Liniński. Usefulness of Tc-99m-N,N'-ethylene-1-dicycysteine complex for dynamic kidney investigations. *Nucl. Med. Comm.* 15:628–635 (1994).
23. S. Ratner and H. T. Clarke. The action of formaldehyde upon cysteine. *J. Am. Chem. Soc.* 59:200–206 (1937).
24. P. Blondeau, C. Berse, and D. Gravel. Dimerization of an intermediate during the sodium in liquid ammonia reduction of L-thiazolidine-4-carboxylic acid. *Can. J. Chem.* 45:49–52 (1967).
25. M. P. Hay, W. R. Wilson, J. W. Moselen, B. D. Palmer, and W. A. Denny. Hypoxia-selective antitumor agents. Bis(nitroimidazolyl)alkanecarboxamides: a new class of hypoxia-selective cytotoxins and hypoxic cell radiosensitizers. *J. Med. Chem.* 37:381–391 (1994).
26. A. Cherif, D. J. Yang, W. Tansey, E. E. Kim, and S. Wallace. Synthesis of [<sup>18</sup>F]fluoromisonidazole. *Pharm. Res.* 11:466–469 (1994).
27. G. V. Martin, J. H. Cardwell, M. M. Graham, J. R. Grierson, K. Kroll, M. J. Xowna, T. K. Lewellen, J. S. Rasey, J. J. Casciari, and K. A. Krohn. Noninvasive detection of hypoxic myocardium using [<sup>18</sup>F]fluoromisonidazole and PET. *J. Nucl. Med.* 33:2202–2208 (1992).
28. S. H. Yeh, R. S. Liu, H. H. Hu, C. P. Chang, L. S. Chu, K. L. Chou, and L. C. Wu. Ischemic penumbra in acute stroke: demonstration by PET with fluorine-18 fluoromisonidazole. *J. Nucl. Med.* 35:(5)205 (Abstract) (1994).
29. S. H. Yeh, R. S. Liu, L. C. Wu, D. J. Yang, S. H. Yen, C. W. Chang, T. W. Yu, K. L. Chou, and K. Y. Chen. Fluorine-18 fluoromisonidazole tumor to muscle retention ratio for the detection of hypoxia in nasopharyngeal carcinoma. *Eur. J. Nucl. Med.* 23:1378–1383 (1996).
30. R. S. Liu, S. H. Yeh, C. P. Chang, L. S. Chu, M. T. Lui, K. L. Chou, and L. C. Wu. Detection of odontogenic infections by [<sup>18</sup>F]fluoromisonidazole. *J. Nucl. Med.* 35:113 (Abstract) (1994).