Cancer Chemother Pharmacol (2001) 48: 177–187
DOI 10.1007/s002800100324

ORIGINAL ARTICLE

C.J. Koch · S.M. Hahn · K. Rockwell Jr. · J.M. Covey
W.G. McKenna · S.M. Evans

Pharmacokinetics of EF5 [2-(2-nitro-1-*H*-imidazol-1-yl)-*N*-(2,2,3,3,3-pentafluoropropyl) acetamide] in human patients: implications for hypoxia measurements in vivo by 2-nitroimidazoles

Received: 27 November 2000 / Accepted: 30 January 2001 / Published online: 27 June 2001

Abstract *Objectives*: Pharmacokinetic studies were performed on the first 28 patients enrolled in a phase I trial to determine the ability of EF5 [2-(2-nitro-1-*H*-imidazol-1-yl)-*N*-(2,2,3,3,3-pentafluoropropyl) acetamide] to detect hypoxia in human tumors in the absence of patient toxicity. *Methods*: EF5 was made in purified form and formulated for intravenous injection by the National Cancer Institute. After obtaining consent from the patients, EF5 was administered and blood samples were drawn at various times over approximately 48 h. For most patients it was possible to collect total urine at approximately 8-h intervals. EF5 in plasma and urine was analyzed by high-performance liquid chromatography. *Results*: EF5's plasma concentration followed a simple exponential decay following infusion. The plasma half-life was 11.7 ± 2.6 h ($\pm$SD) and was not affected by drug dose (9 to 28 mg/kg), fractional urine recovery, patient weight or gender. Absolute plasma values suggested even biodistribution of the drug throughout the soft tissue with a volume of distribution equal to 0.56 l/kg. Despite the relatively high lipid partition coefficient ($\log P = 0.6$), EF5 was excreted primarily (up to 70%) via kidney clearance. No drug metabolites (e.g. retaining the 2-nitroimidazole chromophore) were detected in either plasma or urine. No toxicity was found at drug doses adequate to detect tumor hypoxia. *Conclusions*: Currently held paradigms of 2-nitroimidazole metabolism (e.g. clearance rate and toxicity as affected by octanol/water partition coefficient) are discussed. The results reported herein suggest that EF5 is biologically stable with predictable pharmacokinetics. EF5's consistent half-life and clearance properties will allow quantitative analysis of EF5 binding relative to tissue oxygen levels.

Keywords Predictive assays · Hypoxia detection · Radiation sensitizers · Plasma half-life · Renal clearance

C.J. Koch (✉) · S.M. Hahn · K. Rockwell Jr.
W.G. McKenna · S.M. Evans
University of Pennsylvania School of Medicine,
Radiation Oncology, Philadelphia,
PA 19104-6072, USA
E-mail: kochc@mail.med.upenn.edu
Tel.: +1-215-8980073
Fax: +1-215-8980090

K. Rockwell Jr.
University of Pennsylvania School of Medicine,
Investigational Drug Service,
Philadelphia, PA, USA

J.M. Covey
Toxicology and Pharmacology Branch,
Developmental Therapeutics Program,
National Cancer Institute, Bethesda, MD, USA

Introduction

2-Nitroimidazoles have been under continuous development for more than 25 years, first as hypoxic cell radiosensitizing agents, then as hypoxic cell cytotoxins and more recently as hypoxic cell markers (see references 1, 2, and 5 for reviews). The initial demonstration of the third property, hypoxic cell marking, was by Varghese and Whitmore [53] but the main thrust of development came from Chapman and colleagues in the early 1980s [6, 7]. Several 2-nitroimidazole drugs are being tested for their ability to detect tumor hypoxia via the known ability of such drugs to undergo hypoxia-dependent metabolism. This metabolism leads to the formation of cellular covalent drug adducts at a rate which is maximal in the absence of oxygen and which decreases as a function of oxygen concentration [6, 29]. Drug adducts must then be detected by various means in order to make a biological assessment of hypoxia. A number of detection strategies have been used, including invasive and noninvasive radioactivity measurements [8, 22], NMR/MRI [38], polyclonal antibodies [24, 39] and monoclonal antibodies [33].

Several drugs were formulated by taking a given drug with relatively known metabolic characteristics and adding a detection moiety either covalently or through chelation. Such additions were often unstable in vivo

(e.g. dehalogenation in the liver [26]) leading to a separation of the marker from its detection moiety. The resulting independent biodistribution and metabolism of markers and their detecting chemical group suggests the need for further optimization of clinically useful agents. Based on data from the sensitizer trials, lipophilic drugs were most susceptible to nonoxygen-dependent gut and liver metabolism and, in addition, caused toxicity at high doses. Thus, most new drug development emphasized highly polar compounds, especially for noninvasive hypoxia assays [8, 55]. Polar compounds were expected to be cleared rapidly by the renal system and to be excluded from nervous tissue (the site of probable toxicity).

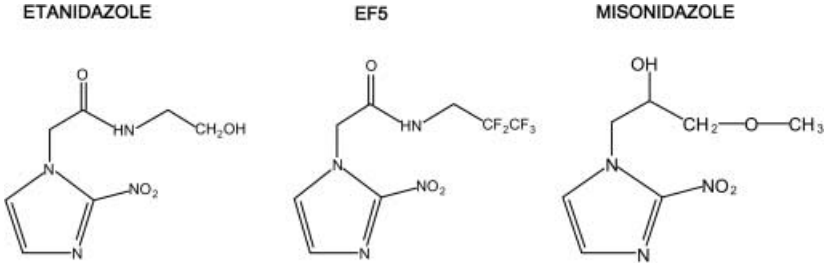
Metabolism of the 2-nitroimidazoles is thought to occur via multiple one-electron reduction steps (see reference 28 for review). The oxygen dependence of metabolism arises at the one-electron reduction stage where the nitro-radical anion can either donate its unpaired electron to oxygen, thus reversing the reduction process, or undergo further reduction. Cellular adducts (predominantly to thiol residues) occur at the two (nitroso) or four (hydroxylamine) electron reduction stage [34]. Despite the apparent similarity in mechanism for the metabolism of various nitroimidazoles, the absolute rate and kinetics of oxygen-dependent inhibition of metabolism differ markedly for various compounds. In addition, there can be substantial variations in metabolism from one cell type to the next. This is an undesirable property for the use of such markers as absolute indicators of tissue oxygenation [28]. For example, early studies emphasized the metabolism of misonidazole. In vitro, the absolute rate of binding, the kinetics of the oxygen dependence of binding, and the influence of other redox-modulating drugs suggested that it would not be possible to use misonidazole for quantitative analysis of tissue oxygen levels [28]. In vivo, work often focused on attempting to explain the distribution of high binding in normal murine tissues, e.g. leading to the suggestion that normal liver and esophagus were severely hypoxic [9]. Pharmacological studies demonstrated the rapid breakdown of many 2-nitroimidazoles (e.g. elimination of terminal methyl of misonidazole) by mechanisms that could not be explained by hypoxia but which involved 'nonhypoxia-dependent' metabolism by kidney, liver and gut (see reference 58 for review).

In 1981 a new 2-nitroimidazole (SR-2508, etanidazole) was introduced for use as a clinical hypoxic cell radiosensitizer. This compound was designed, by virtue of its very high polarity, to have a relatively short biological half-life and to be excluded from brain and other nervous tissues, thus reducing the predominant toxicity of such compounds – peripheral neuropathy [3]. In fact, etanidazole is so polar that it preferentially partitions outside cells in vitro [30]. In vivo, etanidazole is much less neurotoxic than its predecessors, and is excreted predominantly via the kidneys without other metabolites [11]. As a hypoxia marker, etanidazole has been found to be free of the problems previously associated with misonidazole and its derivatives [28]. Unfortunately, the high drug polarity leads to a relatively low cellular uptake [30, 37] and there are no suitable detecting moieties.

The unique stability of etanidazole in vivo can only partly be explained on the basis of its rapid clearance. We reasoned that another cause may be the specific sidechain structure. Therefore, a new drug with a sidechain similar to that of etanidazole was synthesized for the principle purpose of hypoxic cell detection (in cooperation with Drs. Mike Tracy and Robert Sutherland, SRI International). Specifically, the terminus of etanidazole's sidechain was modified by substituting the terminal $-CH_2OH$ by $-CH_2CF_2CF_3$ (Scheme 1). This drug, EF5 [2-(2-nitro-1-*H*-imidazol-1-yl)-*N*-(2,2,3,3,3-pentafluoropropyl) acetamide], was rationally designed to have the following properties:

1. C–F is the most stable chemical bond. Thus, EF5 should retain or surpass etanidazole's exceptional stability in vivo.
2. Since C–F bonds do not occur naturally, EF5's $-CF_2CF_3$ terminus should provide an excellent hapten for the development of monoclonal antibodies (MAb) against the drug and its adducts.
3. The $-CF_2CF_3$ terminus should make EF5 significantly more lipophilic than etanidazole, allowing uniform drug access to all tissues including brain in vivo. Such access was deemed essential to the development of an assay system which could be used independently of tissue type or location.
4. The enhanced lipophilicity of EF5 was expected to increase its biological half-life.
5. The enhanced lipophilic character of EF5 was expected to allow enhanced signal from hypoxia-dependent metabolism [28].
6. Fluorine atoms are paramagnetic, potentially allowing detection of EF5 metabolism by nuclear magnetic resonance (NMR).

Scheme 1



7. [^{18}F]Fluorine is a positron emitter, potentially allowing detection of EF5 metabolism by positron emission tomography (PET).
8. The combination of items 3 and 4 was anticipated to make EF5 more neurotoxic than etanidazole, since it would have full access to the brain and nervous tissue. However, we intended EF5 to be used at much lower drug exposures than was etanidazole (i.e. a diagnostic rather than a therapeutic drug).
9. The high lipophilicity of EF5 was expected to substantially diminish the relative importance of kidney clearance compared to etanidazole. Clearance in the gut could cause imaging problems in this part of the body.

Remarkably, all of the above predictions regarding EF5's chemical and biological properties have been confirmed, with the exception of the two problematic ones – items 8 and 9 – each of which could have interfered with the clinical development of this drug. In humans, EF5 appears to be nontoxic at the doses used in this study and is cleared predominantly by the kidney. The results below detail phase I studies of the pharmacokinetics of EF5 in human cancer patients.

Materials and methods

Sample preparation

In our initial studies of plasma or whole blood pharmacokinetics in rodent and other species [32] performed under non-GLP conditions, a simple acid precipitation of macromolecular species from blood or plasma was used since EF5 is stable in 5% trichloroacetic acid (TCA). Subsequent development [23a] resulted in a solid-phase extraction procedure, with twofold higher sensitivity, using C18 SepPak columns. This method was validated in human plasma and found to be linear with a detection limit of 7.5 pmol. As described below, the simpler acid-precipitation method was also suitable for quantitative recovery of EF5 from blood, and we have continued with this method.

HPLC conditions

Ammonium acetate (100 m*M*, pH 4.6) was dissolved in a mixture of 40% MeOH plus water and was continuously sparged with helium. Solvent flow at a rate of 1 ml/min was maintained by a Waters 510 pump, through a cooled autosampler (Waters 712WISP) and then into an Alltima C18 column from Alltech (4.6×250 mm, ID×length) with a guard column of the same material. The temperature was maintained at 30°C by a Waters temperature control module. Solvent flow from the column was fed into a Waters 490 four-channel spectrophotometer monitored by a chart recorder (The Recorder Company, Houston, Tx.). EF5 eluted at 11.2 ± 0.5 min under the above conditions. The variation in retention time is accounted for by changes over the life of a column. On any given run, the elution time of the EF5 drug peak was within a few seconds (data not shown). The absorption peak for EF5 is in the range 325–327 nm and the absorption coefficient, using recrystallized drug, was determined to be $7900/M$/cm.

In the absence of a suitable standard incorporated into the acid, we programmed the autosampler to make two standards (prepared using authentic drug made by contractors for the NCI under GMP conditions) for every four samples. All samples were prepared and/or diluted by mass, with accuracy to 0.1%. Reproducibility of standard peak heights was typically 0.5%. Sample volumes were typically 75 μl.

The assay was validated by diluting EF5 into 5% TCA or fetal calf serum, with storage at 4°C. The serum samples were then individually diluted with an equal mass of 10% ice-cold TCA (densities for both are almost the same) on day 1, 2 or 3 of storage. After centrifugation to remove precipitated macromolecules, the sample supernatants were subjected to HPLC the same day. Biological stability was tested by preparing similar dilutions in fresh heparinized human blood. The first set of dilutions was prepared immediately (by adding an equal mass of 10% TCA) while the remainder of each blood sample was stored (with agitation by a Thermolyne Varimix rocker) overnight at room temperature. A second set of dilutions was then prepared from the stored blood.

Patient samples

Since the EF5 concentration in the i.v. injection fluid was only 9.9 m*M* and the infusion rate was limited to 350 ml/h, there was a substantial range (because of patient mass and drug dose) in the total infusion time (0.75 to >2.5 h). Because of the long plasma half-life (see below) corrections for the infusion time were not made. Therefore, the timing of sampling was defined with respect to the start of the patient infusion: blood was drawn into heparinized tubes immediately before drug infusion, 1 h following the completion of the infusion, 3–4 h later before the patient left the hospital, during a visit the following day, and just preceding surgery on day 2. Some patients were unable to return to the hospital for blood sampling the following day. A preinfusion urine sample was followed by collection of total urine for the approximately 48 h from infusion start until surgery.

Urine samples (volume estimated by mass) included the first while the patient remained at the hospital, then others in 8–12 h increments until surgery. Since each urine sample represented the time average of renal output, the 'time' assigned to each urine sample was calculated from the start of drug infusion to the midpoint of the collection interval. Blood in original collection tubes and 4-ml samples of urine were centrifuged at 300 *g* for 12 min. Each urine supernatant or plasma sample was stored at 4°C until it could be diluted in duplicate into 1.5-ml polypropylene microcentrifuge tubes with an equal mass of 10% TCA. Pooled acid plus sample was shaken, tubes stored for at least 30 min on ice, then spun at maximum speed on a Fisher 59A microcentrifuge. The clear supernatants were then added to 0.3-ml autosampler tubes.

The duplicate dilutions of each plasma and urine sample were analyzed in two independent HPLC runs, providing four separate measurements of EF5 concentration for each patient sample. For every four patient samples, a primary standard and a 4:1 dilution of the primary standard were assessed. Two additional dilutions of the primary standard (20:1, 100:1) were also included as samples. After ensuring that recorder peak heights accurately reflected drug concentration (i.e. peak integration was not necessary – see below) peak heights were measured from the chart recorder output by hand. Drug concentrations in the original samples were calculated in a spreadsheet by comparing peak heights of sample to that of the primary standard and allowing for the dilution.

The clinical trial was designed to escalate drug concentration with six patients at each drug level. Since toxicities were not expected, based on preclinical studies in rats and dogs (unpublished NCI data; 23a), the criteria for escalation were based on the maximum expected binding signal for a given AUC [19]. The drug levels planned were 9, 12, 16, 21 and 28 mg/kg. All toxicities were monitored. Any two grade 3 or 4 toxicities would prevent going to the next drug level or require a de-escalation. If toxicities were found at the lowest planned drug level, de-escalation possibilities were 6 and 4 mg/kg. The starting dose (9 mg/kg) was chosen to provide an AUC roughly equivalent to that in former rodent studies, while recognizing that EF5's metabolism by human tumor cells was likely to be about threefold lower than that by rodent tumor cells.

Results

Actual HPLC output for drug standard, and control vs drug-containing human plasma or urine showed no interfering peaks within ±4 min of the peak for EF5 (Fig. 1). As can be seen from a series of standards made in either physiological saline or calf serum, followed by 1:1 mixing with 10% TCA and analysis, the peak height increased linearly with EF5 concentration and was highly reproducible on a day-to-day basis (Fig. 2). The detection limit was 0.2 μ*M* for a 75-μl injection volume (15 pmol). No changes in standard were observed when stored for up to 2 months at 4°C in dim light (data not shown). Similar linearity was observed for samples prepared in whole blood or urine. EF5 (0.5 to 80 μ*M*) was also stable in blood for 16 h at room temperature (data not shown).

Since 2-nitroimidazoles with high lipophilicity had not previously been administered to humans, it seemed important to obtain some preliminary information on the possible drug retention time in humans before commencing the clinical trial. Thus in the tradition of the early sensitizer trials [21], a small amount of drug was ingested orally by one of the authors (C.K.). Urine was collected for 2 days at roughly 4-h intervals and analyzed as described above. Drug levels in urine decreased in an approximately monoexponential fashion with a half-life about 12 h, and about 40% was recovered in unaltered form with no additional UV peaks (Fig. 3).

For the first two patients, i.v. drug administration at 9 mg/kg gave similar fractional urine recoveries (42 and 44%) compared with the recovery from the much smaller oral dose described above (Fig. 4). Since drug delivery was slow and the number of blood samples limited, the biodistribution phase of drug from plasma

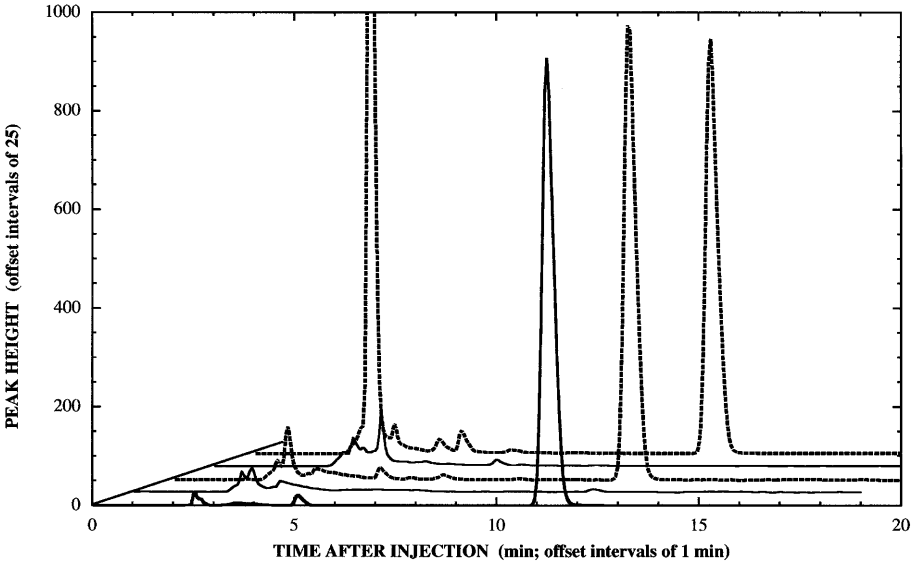


Fig. 1 Representative HPLC chromatograms for EF5 analysis. To allow easy visualization, the four experimental traces are each displaced (by 1 min and 25 peak height) from the standard (foremost curve along the *x*-axis) to create a three-dimensional effect. The order of the four experimental curves is: plasma, control; plasma, EF5-treated; urine, control; urine, treated. The tiny peak at the expected EF5 elution for the control plasma (11.3 min after injection) was from trace carryover of the standard from the autoinjector – in this case 1.0 m*M* (appropriate for urine samples) rather than the normal 0.1 m*M* used for plasma analysis

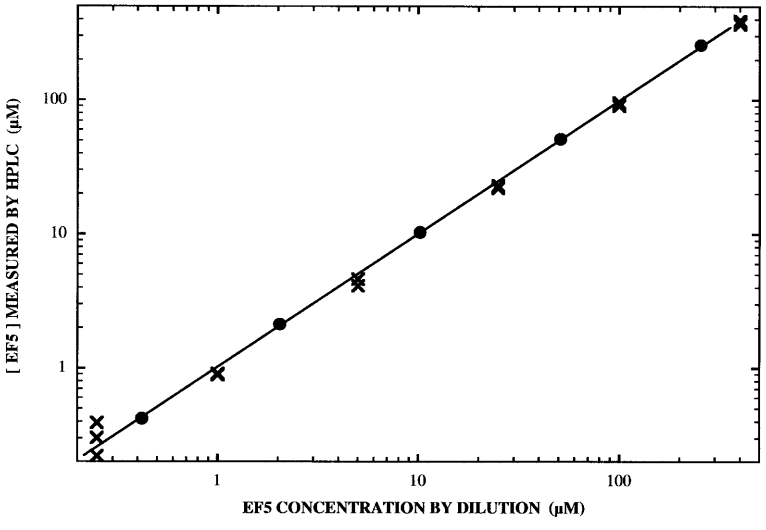


Fig. 2 Test of recovery of EF5 from calf serum after acid precipitation of samples and HPLC analysis on days 1, 2 and 3 following initial drug dilutions. Below 1 μ*M*, variability occurs primarily due to detector noise and/or recorder dead space. The correlation coefficient of the straight line through the points has an *r*-value greater than 0.99

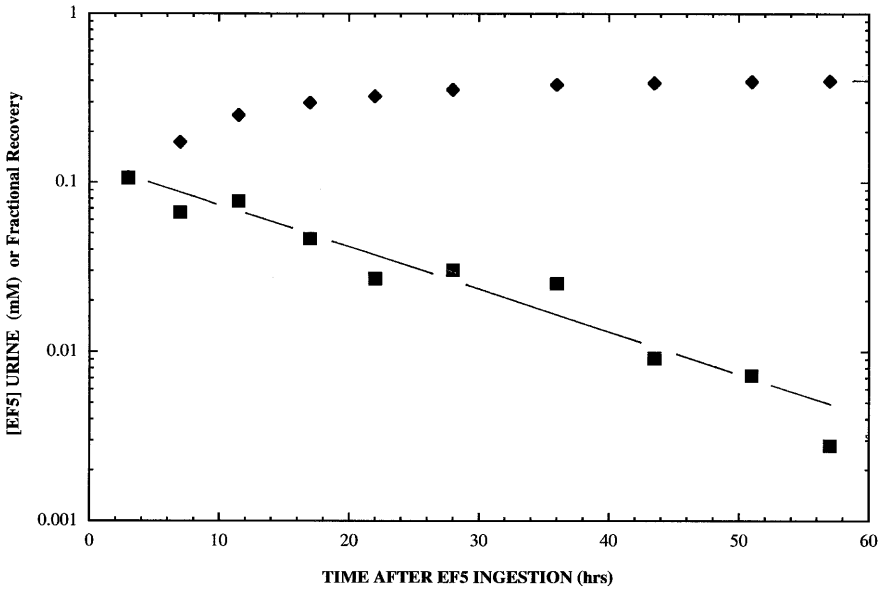


Fig. 3 Analysis of EF5 in urine using the methods described after a small oral dose (2 mg/kg) by one of the investigators. The *y*-axis scale has a dual meaning: fractional urinary recovery (*diamonds*, dimensionless) and urine concentration (*squares*, m*M*)

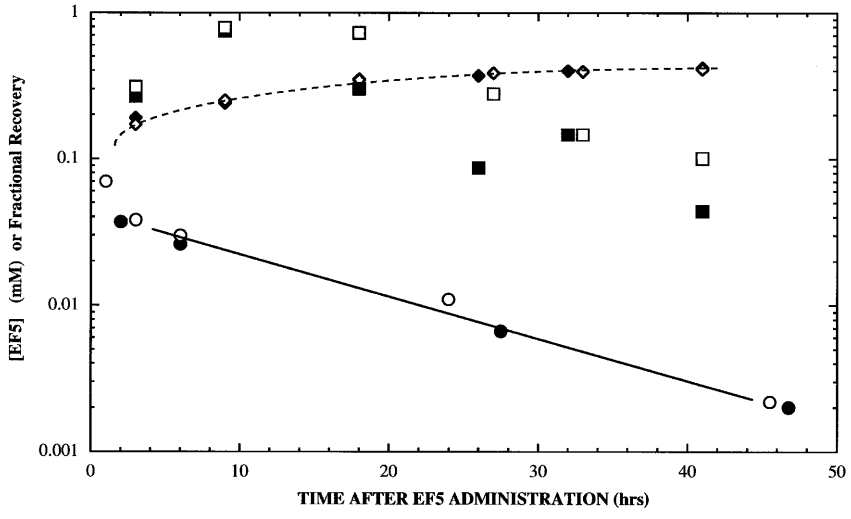


Fig. 4 Plasma and urine pharmacokinetic decay of EF5 for the first two patients of the study treated with an EF5 dose of 9 mg/kg. Each point represents the average of four determinations, with errors much smaller than the points as plotted (*open symbols* patient 1, *closed symbols* patient 2; *circles* plasma concentration, m*M*; *squares* urine concentration, m*M*; *diamonds* fractional urinary recovery, dimensionless)

was not measured. At times greater than 1 h following completion of drug administration, plasma decay was found to be first order, and after 4–6 h the curve describing urine concentration paralleled that of plasma concentration (Fig. 4). Thus, a log-linear fit of the line describing plasma decay (ln[EF5] vs time) provided an estimated equilibrium concentration at time zero (to calculate volume of distribution) and plasma half-life. Integration of this curve from time zero to the data point taken at surgery (tissue sampling) provided the total drug exposure (AUC, m*M*·h).

There was essentially no change in EF5's plasma half-life with drug dose (Fig. 5), and the average value of 11.7 ± 2.6 h was not different from that calculated from the urine decay of the small oral dose represented in Fig. 3 (the outlier at >24 h was ignored in this average; including it gives 12.1 ± 3.5 h). Similarly, there was no significant change in drug plasma half-life with patient gender or age (data not shown), although there was a slight trend towards a positive correlation with the latter. Extrapolated equilibrium concentration at zero time and AUC were proportional to injected dose (Fig. 6), although there was some indication that AUC was more variable at the two highest drug doses (21 and 28 mg/kg). The initial drug concentration can be calculated by taking the administered dose and assuming a volume of distribution of 0.56 ± 0.06 l/kg. The dose escalation study was terminated at four patients for the highest drug level (28 mg/kg) based upon an episode of increasing confusion in a patient with a large recurrent glioblastoma multiforme and one unexplained episode of grade 1 hypotension. The former was thought to be explained by the large fluid volume.

There was no correlation between fractional urine recovery, which varied from 0.17 to 0.71, and injected dose (Fig. 7). There was also no correlation between fractional urine recovery and plasma half-life or AUC (data not shown). Expected toxicities from 2-nitroimi-

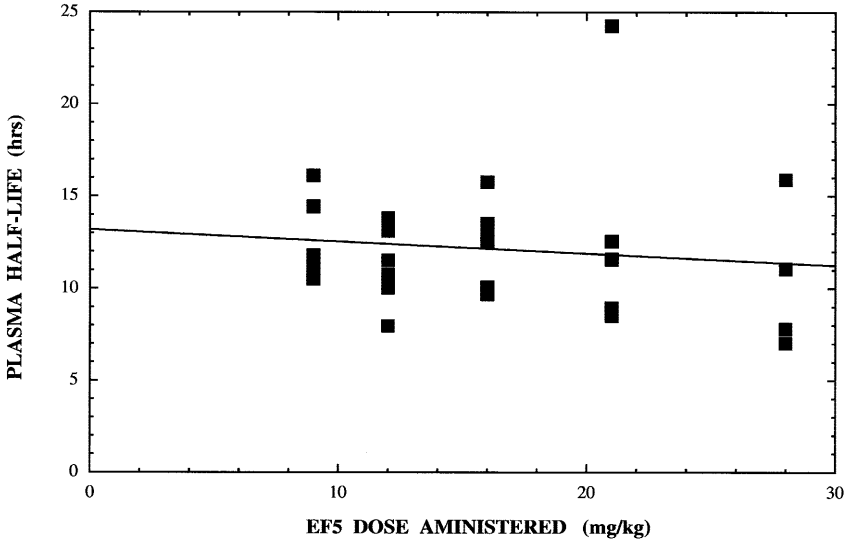


Fig. 5 Summary of plasma half-life for all patients studied at administered doses of 9, 12,16, 21 and 28 mg/kg. Data are summarized for 27 of the 28 patients

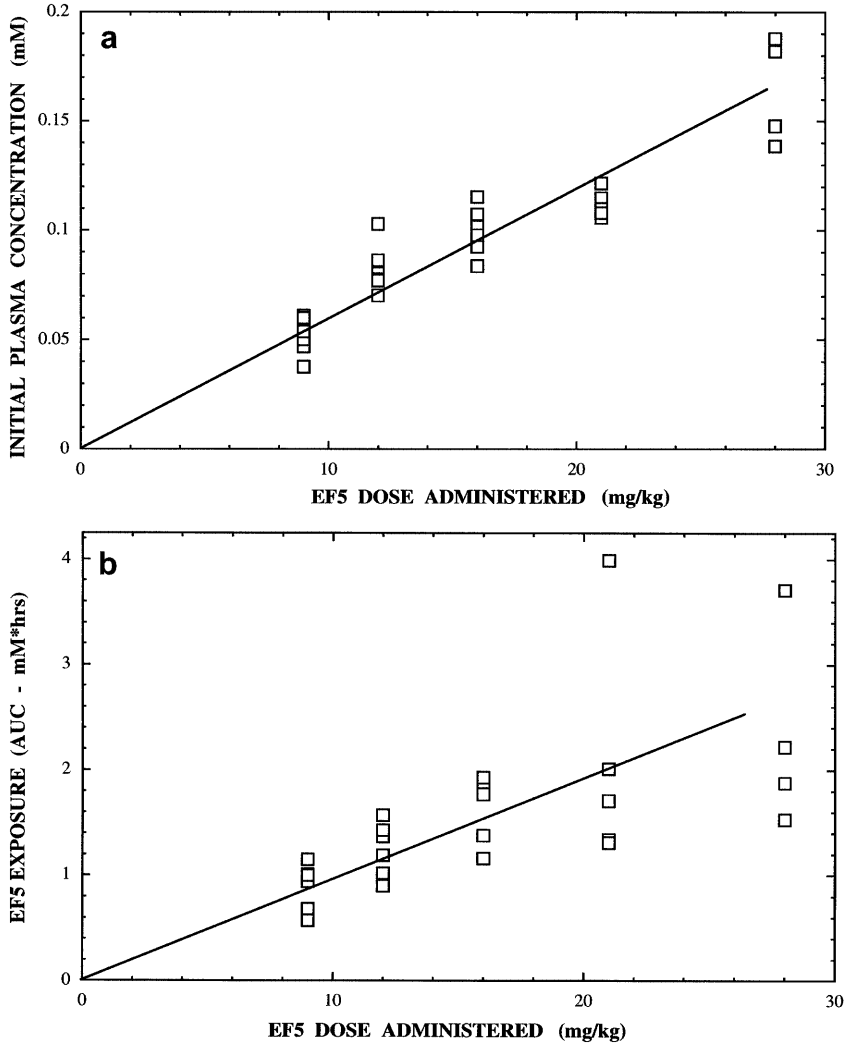


Fig. 6 **a** Variation in extrapolated initial plasma concentration with dose (all 28 patients) ($r = 0.93$, $P < 0.001$). **b** Variation in AUC with injected dose (27 of 28 patients) ($r = 0.66$, $P < 0.001$)

dazoles (nausea, flushing, rash, and peripheral or central neuropathies) were not found.

A summary of all kinetic parameters, summarized for all patients at each drug dose, is provided in Table 1.

Discussion

As indicated in the Introduction, there are only a few examples of 2-nitroimidazoles designed specifically as hypoxia-imaging agents. The expected properties of EF5 listed in the Introduction indicate the need for (1) biochemical stability, (2) consistent pharmacokinetics, (3) lack of toxicity, (4) uniform biodistribution and (5) consistent rate and oxygen dependence of metabolism. These properties have been observed for EF5 (although uniform biodistribution has not yet been proven in humans – see below) and can be discussed in the context of other nitroimidazoles (see Table 2). Although most of the compounds listed in Table 2 were studied as therapeutic rather than diagnostic agents, most pharmacokinetic parameters (other than toxicity) were relatively independent of total drug dose.

Drug stability is a critically important parameter in translation of chemical and in vitro results to the animal or clinical setting. Although a high percentage renal clearance of parent drug is a good positive indicator of in vivo drug stability the converse is not necessarily true since the fraction of drug cleared and/or metabolized in the gut can be unpredictable and highly species-specific. While detailed HPLC data are not available for all compounds shown in Table 1, EF5 is clearly among the most stable of the 2-nitroimidazoles studied to date. Despite its relatively high lipophilicity, EF5's renal clearance is as high as the much more polar etanidazole and desmethylmisonidazole. EF5 is extremely resistant to chemical attack other than bioreduction of its nitro group, the desired oxygen-dependent metabolism. Its pharmacokinetic properties have been evaluated in many species and, to date, we have seen no metabolites which retain the nitro chromophore (absorbance at 326 nm) in blood, urine or tissues (e.g. see Fig. 1). In rats and mice, similar experiments have been performed with radioactive drug (label at ^{14}C-2-position of the nitroimidazole ring) with no evidence of labeled breakdown products [32].

Recently, we have achieved labeling of the sidechain with ^{18}F, both for EF5 [16] and a polar analog (EF1, with only one fluorine atom on the terminal propyl group of the sidechain) [18]. Both sidechain labels also show complete stability in vivo (data not shown). In addition, the synthesis procedure for ^{18}F-labeled EF5 requires boiling the radiolabeled product in trifluoroacetic acid – a condition which has no effect on the

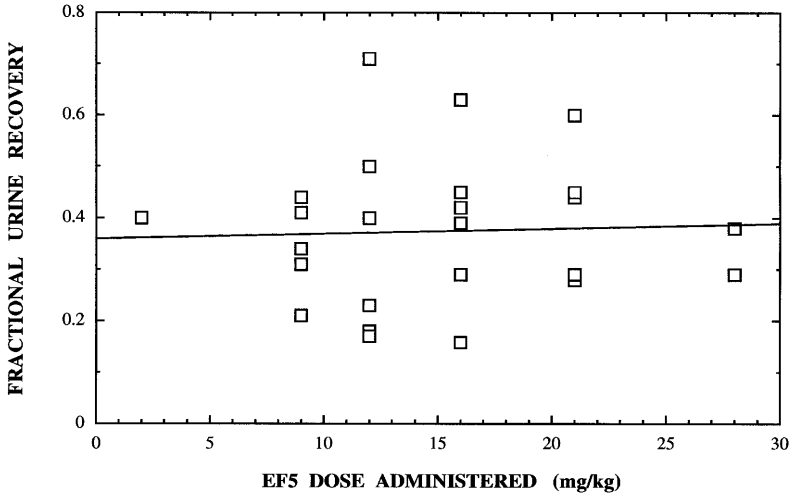


Fig. 7 Lack of dependence of fractional urine recovery on injected dose (25 of 28 patients)

Table 1 Summary of pharmacokinetic parameters for individual patient groups based on dose. Group sizes were one for the oral dose, six for 9–21 mg/kg and four for 28 mg/kg. Fractional renal clearance (CL) was not available for one patient from each of the thee groups, and plasma clearance time was not available for one patient. Overall CL was 0.37 ± 0.14 and overall V_d was 0.56 ± 0.08 l/kg. Overall plasma half-life was11.7 ± 2.6 h or 12.1 ± 3.5 h in the absence and presence (respectively) of one outlier (24.3 h)

Dose (mg/kg)	Route of administration	Plasma half-life (h)	Initial plasma concentration (mM)	AUC (mM·h)	V_d (l/kg)	CL
2	Oral	12				0.40
9	Injected	13.0 ± 2.2	0.052 ± 0.009	0.89 ± 0.22	0.59 ± 0.11	0.34 ± 0.09
12	Injected	11.2 ± 2.1	0.083 ± 0.011	1.25 ± 0.26	0.49 ± 0.06	0.36 ± 0.21
16	Injected	12.4 ± 2.3	0.100 ± 0.011	1.66 ± 0.31	0.55 ± 0.06	0.39 ± 0.16
21	Injected	13.2 ± 6.4	0.111 ± 0.005	2.07 ± 1.11	0.63 ± 0.03	0.41 ± 0.13
28	Injected	10.5 ± 4.0	0.164 ± 0.024	2.33 ± 0.96	0.57 ± 0.09	0.35 ± 0.05

Table 2 Summary of toxicological and pharmacological properties for a number of nitroimidazoles used for therapeutic and diagnostic use. The mean tolerated dose (MTD) for single (col 3) or multiple (col 5) doses is normalized to a 70 kg patient with surface area 1.8 square meter. The partition coefficient (*P*) for pimonidazole applies to the neutral form of the drug; it is much lower for the charged form

Drug	*P*	MTD Single dose (g)	$t_{1/2\beta}$ (h)	MTD Multi dose (g)	AUC (mM·h)	Urine (% parent)	References
Metronidazole	1.0	12	7.9	22×2.3	77	13–33	48, 50
Nimorazole	1.4	6	3.5	20×3	>23	None	36, 49
Misonidazole	0.43	10	9.3	10×2	25	3.5–12.5	14, 23, 45
Desmethylmisonidazole	0.11	2	4.9	10×2	11	30–70	10, 15, 47
Pimonidazole	8	1.2	5.8	10×1.2	7.5	10–36	38, 42, 43
Etanidazole	0.05	32	5.5	20×3	35	25–61	11, 13, 41
EF5	4.0	2.0[a]	11.6	NA	4[a]	23–71	This report
Ku-2285	1.5	12	ND	25×2	ND	ND	46

[a]Toxicity was not found at the given dose, in contrast to other compounds listed

molecular structure of EF5. In noninvasive applications of tissue hypoxia imaging it is extremely important that the label stay associated with the drug. Thus, it has recently been demonstrated that fluoromisonidazole forms multiple radioactive peaks in urine and only a relatively small fraction of total drug is cleared renally [40]. Interestingly, these authors showed that a monofluorinated derivative of etanidazole is much more biologically stable, confirming our rationale for the importance of sidechain structure.

Consistent pharmacokinetic properties of drugs used for imaging hypoxia is essential since drug metabolism is linearly related to drug exposure or AUC when other modifying factors, e.g. tissue pO_2, are equal. Drug half-life would be expected to increase with lipophilicity (because of lowered renal excretion) and also with drug stability in vivo [57] and indeed, the rank order of drug plasma half-lives is roughly consistent with drug lipophilicity. This relationship is quite weak, however, with an 80-fold increase in partition coefficient (between etanidazole and EF5) resulting in only a 2-fold increase in plasma half-life. The half-lives of nimorazole and pimonidazole are most discrepant in this regard. Nimorazole has an unexpectedly short half-life in vivo because of its failure to survive renal clearance (note that urine recovery of parent drug is nil). We have been unable to find the source of this drug instability in literature reports [36, 49]. Similarly, pimonidazole undergoes a reversible oxidation of its sidechain, thereby reducing the concentration of parent drug [54]. Furthermore, the high lipid partition coefficient listed for pimonidazole in Table 1 is for its uncharged form. The sidechain of pimonidazole is a weak base so a portion of the circulating drug is always in a charged, highly polar form which should be cleared more rapidly [38, 42, 43]. EF5 has the longest half-life of the nitroimidazoles tested in humans. Despite EF5's lipophilic character, up to 70% of the drug is cleared renally.

The initial plasma concentration of EF5 can be predicted accurately by drug dose (assuming a volume of distribution of 0.56 l/kg), so AUC variation is caused primarily by patient-to-patient variations in drug half-life. EF5's plasma half-life, determined in the present studies was, with the exception of one outlier, 11.7 ± 2.6 h ($\pm$SD). Variations in drug clearance rates are common in patients with serious disease. The source of these variations is under investigation but they do not appear to be related to recovery of drug in urine, under the limitations of patient compliance with total urine collection. Variations in AUC may be accounted for by obtaining a plasma concentration at the time of tissue assay since plasma clearance was consistently first order. At 21 mg/kg, EF5 was well-tolerated and its initial plasma concentration was 0.111 ± 0.005 m*M* ($\pm$SD).

An alternative approach to obtaining a consistent AUC in the face of a somewhat variable plasma half-life is to use drug exposure times much less than the drug half-life, where the AUC will be dominated by initial drug concentration. This approach is being used by investigators in Europe using another hypoxia-detecting 2-nitroimidazole, pimonidazole (B. van der Kogel, personal communication). Tumor biopsy samples are being taken from patients 2–3 h after drug administration. This procedure may be somewhat more convenient for patients, but will substantially reduce the maximum possible drug-adduct signal (related to AUC), especially since (a) pimonidazole is being used at lower doses than EF5 and (b) only about half of the drug is available for binding due to metabolism of the sidechain in vivo [42, 54]. In the European trial, tissue sampling is being limited to tissue biopsies. For macroscopic surgical biopsies (as obtained in the EF5 trial), the approach of using short drug exposure times could lead to substantial variability due to modifications in tissue blood flow caused by anesthesia and devitalization of the tumor blood supply during surgical excision.

Toxicity from 2-nitroimidazoles, determined in the sensitizer trials, has been typically characterized by peripheral or central neuropathy [12, 35, 51]). The concept established by Bleehen, Workman and colleagues in the1970s and 1980s is that nervous system toxicity is related to total drug exposure (AUC) in this tissue [42]. Thus, most subsequent drug development has emphasized highly polar compounds which are excluded from

nervous tissue. For a limited number of nitroimidazoles, drug polarity has been also found to substantially enhance kidney clearance, as discussed above [56]. A partial reduction in the toxicities from nitroimidazoles can be achieved by giving the total drug dose in small daily increments. This is especially true for pimonidazole (a 2-nitroimidazole also being used to detect tissue hypoxia [27]) which has been found to cause a unique syndrome of central neuropathy at single doses exceeding 1 g/m^2 [42]. Pimonidazole was the first sensitizer with high lipophilicity, presumably allowing central nervous system (CNS) access with the attendant CNS toxicity.

Because of its lipophilicity and long plasma half-life, we expected EF5 to be a relatively toxic drug. Considering that pimonidazole has a twofold shorter half-life than EF5, and that only half of the drug circulates in unaltered form, we anticipated that CNS toxicity might be observed for EF5 at lower doses than the 1 g/m^2 found for pimonidazole. Counterbalancing this concern was the complete lack of nervous (or any other) tissue damage noted for EF5 in canine preclinical studies, even at total drug exposures tenfold higher than the maximum predicted for the patient studies reported here (unpublished data from IND application to the FDA). The actual observations in this phase I study of EF5 showed none of the toxicities expected for 2-nitroimidazoles [19, 20]. The minor toxicities noted in the Results were most likely related to the drug infusion volume and no toxicities of any sort were noted at up to 21 mg/kg. Lack of toxicity is of critical importance for a diagnostic drug. It is of interest that other fluorinated 2-nitroimidazoles being considered for therapeutic use in Japan (e.g. Ku-2285) have also proven quite nontoxic [46]. Accepting the nervous system access paradigm, one would have expected that desmethylmisonidazole (a polar metabolite of misonidazole) would have been very nontoxic, since it is almost as polar as etanidazole. In fact, based on AUC, desmethylmisonidazole is among the most toxic sensitizers yet tested (exceeded only by pimonidazole, see Table 2). Thus, we conclude that central and peripheral neuropathy may depend primarily on drug properties, yet to be determined, other than partitioning.

Uniform tissue access is difficult to assess in patients in the absence of noninvasive imaging methods and/or extensive normal and tumor tissue sampling. Although we have not determined the uniformity of EF5's tissue access in humans, previous results in rodent models [32], coupled with the known drug stability and lipophilicity, suggest uniform access of EF5 to all soft tissues. This is certainly not true for some of the other 2-nitroimidazoles. For example, very polar compounds fail to partition evenly in tissues of varying composition. Thus, tumor concentrations of etanidazole have been shown to vary between 0% (brain tumors) and 250% of plasma [13, 52]. Physical-chemical variations in drug properties can also modify tissue distribution. For example, a unique property of pimonidazole is the weakly basic character of its sidechain, causing concentration in acidic tissues. This property could be useful as a means to enhance maximum signal in acidic tissues (which may be also hypoxic) and has been suggested as a way to improve therapeutic efficiency [43]. However, this may cause a substantial problem for quantitation of tissue AUC, since plasma:tumor ratios for pimonidazole have been shown to vary from less than 1 to 12 [42, 43, 44]. The extent to which such variations in tissue AUC will affect absolute tumor binding levels is presently unknown, but we would predict a direct proportionality between tissue drug concentration and rate of drug metabolism.

In addition to the above-described properties, it is obviously important that a hypoxia detector provide a consistent signal with a dynamic range sufficient to detect a broad range of oxygen levels. At constant oxygen concentration, we have shown that EF5 binding is linearly related to drug exposure (i.e. integrated concentration $\times$ time or AUC) [4] and the signal:noise ratio in rodent cells exposed to 0.3 mM·h is about 100, sufficient to cover the entire physiological range [31]. In all animal tumor models studied, the maximum signal obtained in vivo has been found to be essentially the same as that predicted for severely hypoxic cells exposed in vitro to the same drug exposure as measured from the in vivo pharmacokinetics [4, 17, 25]. In the patients of the presently described studies, we also addressed the potential variation in maximum EF5 binding rate for the various tumors studied [19, 20]. This was assessed by incubating small cube-shaped pieces of tumor tissue under hypoxia with drug, conditions which should allow maximal drug binding to occur. For the tumors investigated, we found that the maximum binding rate varied by a factor of ± 2.5, much smaller than the factor of about 100 which occurs between aerobic and hypoxic tissue. Thus, it appears possible that tissue pO_2, as a continuous variable, could be calculated from quantitative measures of EF5 binding with suitable knowledge of maximum binding rates and drug exposure.

The objectives of the EF5 phase I study were to determine the pharmacokinetic properties of EF5 in human cancer patients in a dose escalation study in which in situ binding of EF5 in patient tumor tissue was simultaneously evaluated. The results reported here show that EF5 is chemically and biochemically stable in humans, in agreement with previous studies in several animal systems. Biodistribution studies in these animal systems suggest that EF5 will have uniform access to all tissues of interest, including brain, allowing studies of tumor biology and normal tissue ischemia. EF5's pharmacokinetic properties in human cancer patients are characterized by a consistent initial plasma concentration, proportional to administered dose and based on a volume of distribution of about 0.56 l/kg. EF5 has a consistent rate of maximal metabolism despite the broad variation in tumor types studied to date. It is nontoxic at concentrations adequate to determine tissue pO_2 over the whole physiological range. Thus, EF5's rational

design has led to a drug which appears ideally suited as a hypoxia marker.

Acknowledgements This work was supported by NIH CA75285, and the Radiation Oncology Department, University of Pennsylvania. The authors would like to thank the many investigators at the NCI who helped in obtaining the IND status for EF5, and who made the drug for human use.

References

1. Adams GE (1990) Selective activation of drugs by redox processes (NATO series A 198). Plenum Press, New York
2. Adams GE, Flockhart IR, Smithen CE, Stratford IJ, Wardman P, Watts, ME (1976) Electron-affinic sensitization VII. A correlation between structure, one-electron reduction potentials and efficiencies of nitroimidazoles as hypoxic cell radiosensitizers. Int J Radiat Biol Oncol Phys 67:9–20
3. Brown JM, Yu NY, Brown DM, Lee WW (1981) SR-2508: a 2-nitroimidazole amide which should be superior tomisonidazole as a radiosensitizer for clinical use. Int J Radiat Oncol Biol Phys 7:695–703
4. Busch TM, Hahn SM, Evans SM, Koch CJ (2000) Depletion of tumor oxygenation during photodynamic therapy: detection by the hypoxia marker EF3 [2-(2-nitroimidazol-1[H]-yl)-N-(3,3,3-trifluoropropyl) acetamide]. Cancer Res S60:2636–2642
5. Chapman JD, Gillespie CJ (1981) Radiation-induced events and their time scale in mammalian cells. In: Lett JT, Adler H (eds) Advances in radiation biology, vol 9. Academic Press, New York, pp 143–198
6. Chapman JD, Baer KA, Lee J (1983) Characteristics of the metabolism-induced binding of misonidazole to hypoxic mammalian cells. Cancer Res 43:1523–1528
7. Chapman JD, Lee J, Meeker BE (1990) Adduct formation by 2-nitroimidazole drugs in mammalian cells: optimization of markers for tissue oxygenation. In: Selective activation of drugs by redox processes (NATO series A 198). Plenum Press, New York, pp 313–323
8. Chapman JD, Engelhardt EL, Stobbe CC, Schneider RF, Hanks GE (1998) Measuring hypoxia and predicting tumor radioresistance with nuclear medicine assays. Radiother Oncol 46:229–237
9. Cobb LM, Nolan J, Hacker T (1992) Retention of misonidazole in normal and malignant tissues: interplay of hypoxia and reductases. Int J Radiat Oncol Biol Phys 22:655–659
10. Coleman CN, Wasserman TH, Phillips TL, Strong JM, Urtasun RC, Schwade JG, Johnson RL, Zagars G (1982) Initial pharmacology and toxicology of intravenous desmethylmisonidazole. Int J Radiat Oncol Biol Phys 8:371–375
11. Coleman CN, Urtasun RC, Wasserman TH, Hancock S, Harris JW, Halsey J, Hirst VK (1984) Initial report of the phase I trial of the hypoxic cell radiosensitizer SR-2508. Int J Radiat Oncol Biol Phys 10:1749–1753
12. Coleman CN, Halsey J, Cox RS, Hirst K, Blaschke T, Howes AE, Wasserman TH, Urtasun RC, Pajek T, Hancock S, Phillips TL, Noll L (1987) Relationship between the neurotoxicity of the hypoxic cell radiosensitizer SR 2508 and the pharmacokinetic profile. Cancer Res 47:319–322
13. Coleman CN, Noll L, Howes AE, Harris JR, Zakar J, Kramer RA (1989) Initial results of a phase I trial of continuous infusion SR 2508 (etanidazole): a Radiation Therapy Oncology Group study. Int J Radiat Oncol Biol Phys 16:1085–1087
14. Dische S, Saunders MI, Lee ME, Adams GE, Flockhart IR (1977) Clinical testing of the radiosensitizer Ro 07-0582: experience with multiple doses. Br J Cancer 35:567–579
15. Dische S, Saunders MI, Stratford MRL (1981) Neurotoxicity with desmethylmisonidazole. Br J Radiol 54:156
16. Dolbier WR, Li A-R, Koch CJ, Shiue C-Y, Kachur AV (2001) [18]F-EF5, a marker for PET detection of hypoxia: synthesis of precursor and a new fluorination procedure. Appl Radiat Isot 54:73–80
17. Evans SM, Jenkins WT, Joiner B, Lord EM, Koch CJ (1996) 2-nitroimidazole (EF5) binding predicts radiation sensitivity in individual 9L subcutaneous tumors. Cancer Res 56:405–411
18. Evans SM, Kachur AV, Shiue C-Y, Hustinx R, Jenkins WT Shiue GG, Karp JS, Alavi A, Lord EM, Dolbier WR Jr, Koch CJ (1999) Non-invasive detection of tumor hypoxia using the 2-nitroimidazole [18F]EF1. J Nucl Med 41:327–336
19. Evans SM, Hahn S, Pook DR, Jenkins WT, Chalian AA, Zhang P, Stevens C, Weber R, Benjamin I, Mirza N, Morgan M, Rubin S, McKenna WG, Lord EM, Koch CJ (2000) Detection of hypoxia in human squamous cell carcinoma by EF5 binding. Cancer Res 60:2018–2024
20. Evans SM, Hahn S, Pook DR, Zhang PJ, Jenkins WT, Fraker D, Hsi RA, McKenna WG, Koch CJ (2000) Hypoxia in human intraperitoneal and extremity sarcomas. Int J Radiat Oncol Biol Phys 49:587–596
21. Foster JL, Flockhart IR, Dische S, Gray A, Lenox-Smith I, Smithen CE (1975) Serum concentration measurements in man of the radiosensitizer Ro-07-0582: some preliminary results. Br J Cancer 31:679–783
22. Garrecht BM, Chapman JD (1983) The labelling of EMT-6 tumors in Balb/c mice with 14C-misonidazole. Br J Radiol 56:745–753
23. Gray AJ, Dische S, Adams GE, Flockhart IR, Foster JL (1976) Clinical testing of the radiosensitizer Ro-07-0582. I. Dose tolerance, serum and tumour concentrations. Clin Radiol 27:151–157

23a. Hill et al (1998) Abstract. Proc Am Assoc Cancer Res 39:521

24. Hodgkiss RJ, Jones G, Long A, Parrick J, Smith KA, Stratford MRL (1991) Flow cytometric evaluation of hypoxic cells in solid experimental tumours using fluorescence immunodetection. Br J Cancer 63:119–125
25. Jenkins WT, Evans SM, Koch CJ (2000) Hypoxia and necrosis in rat (L glioma and Morris 7777 hepatoma tumors: comparative measurements using EF5 binding and Eppendorf needle electrode. Int J Radiat Oncol Biol Phys 46:1005–1017
26. Jette DC, Wiebe LI, Chapman JD (1983) Synthesis and in vivo studies of the radiosensitizer 4-(82Br)bromomisonidazole. Int J Nucl Med Biol 10:205–210
27. Kennedy AS, Raleigh JA, Perez GM, Calkins DP, Thall DE, Novotny DB, Varia M (1997) Proliferation and hypoxia in human squamous cell carcinoma of the cervix: first report of combined immunohistochemical assays. Int J Radiat Oncol Biol Phys 37:897–905
28. Koch CJ (1990) The reductive activation of nitroimidazoles; modification by oxygen and other redox-active molecules in cellular systems. In: Selective activation of drugs by redox processes (NATO series A 198). Plenum Press, New York, pp 237–247
29. Koch CJ, Stobbe CC, Baer KA (1984) Metabolism induced binding of14C-misonidazole to hypoxic cells: kinetic dependence on oxygen concentration and misonidazole concentration. Int J Radiat Oncol Biol Phys 10:1327–1332
30. Koch CJ, Stobbe CC, Baer KA (1986) Combined radiation-protective and radiation-sensitizing agents: III) Radiosensitization by misonidazole as a function of concentrations of endogenous glutathione or exogenous thiols. Int J Radiat Oncol Biol Phys 12:1151–1155
31. Koch CJ, Evans SM, Lord EM (1995) Oxygen dependence of cellular uptake of EF5 [2-(2-nitro-1H-imidazol-1-yl)-N-(2,2,3,3,3-pentafluoroproply) acetamide]: analysis of drug adducts by fluorescent antibodies vs bound radioactivity. Br J Cancer 72:869–874
32. Laughlin KM, Evans SM, Jenkins WT, Tracy M, Chan CY, Lord EM, Koch CJ (1996) Biodistribution of the nitroimidazole EF5 [2-(2-nitro-1H-imidazole-1-yl)-N-(2,2,3,3,3-pen-

tafluoropropyl) acetamide] in mice bearing subcutaneous EMT6 tumors. J Pharmacol Exp Ther 277:1049–1057
33. Lord EM, Harwell LW, Koch CJ (1993) Detection of hypoxic cells by monoclonal antibody recognizing 2-nitroimidazole adducts. Cancer Res 53:5271–5276
34. McClelland RA, Panicucci R, Rauth AM (1985) Electrophilic intermediate in the reactions of a 2-(hydroxylamino)imidazole. A model for biological effects of reduced nitroimidazoles. J Am Chem Soc 107:1762–1763
35. Newman HF, Ward R, Workman P, Bleehen NM (1988) The multi-dose clinical tolerance and pharmacokinetics of the combined radiosensitizers, Ro 03-8799 (pimonidazole) and SR 2508 (etanidazole). Int J Radiat Oncol Biol Phys 15:1073–1083
36. Overgaard J, Overgaard M, Timothy AR (1983) Studies of the pharmacokinetic properties of Nimorazole. Br J Cancer 48:27–34
37. Raleigh JA, Koch CJ (1990) The importance of thiols in the reductive binding of 2-nitroimidazoles to macromolecules. Biochem Pharmacol 40:2457–2464
38. Raleigh JA, Franko AJ, Treiber EO, Lunt JA, Allen PS (1984) Covalent binding of a fluorinated 2-nitroimidazole to EMT-6 tumours in Balb/c mice: detection by F-19 nuclear magnetic resonance at 2.35 T. Int J Radiat Oncol Biol Phys 10:1337–1340
39. Raleigh JA, Miller GG, Franko AJ, Koch CJ, Fuciarelli AF, Kelley DA (1987) Fluorescence immunohistochemical detection of hypoxic cells in spheroids and tumours. Br J Cancer 56:395–400
40. Rasey JS, Hofstrand PD, Chin LK, Tewson TK (1999) Characterization of [18F]fluoroetanidazole, a new radiopharmaceutical for detecting tumor hypoxia. J Nucl Med 40:1072–1079
41. Riese NE, Loeffler JS, Wen PAE, Black PM, Coleman CN (1994) A phase I study of etanidazole and radiotherapy in malignant glioma. Int J Radiat Oncol Biol Phys 29:617–620
42. Roberts JT, Bleehen NM, Walton MI, Workman P (1986) A clinical phase I toxicity study of Ro 03-8799; plasma, urine, tumor and normal brain pharmacokinetics. Br J Radiol 59:107–116
43. Saunders MI, Dische S, Fermont D, Bishop A, Lenox-Smith I, Allen JG, Malcolm SL (1982) The radiosensitizer Ro 03-8799 and the concentrations which may be achieved in human tumours: a preliminary study. Br J Cancer 46:706–710
44. Saunders MI, Anderson PJ, Bennett MH, Dische S, Minchinton A, Stratford MRL, Tothill M (1984) Clinical testing of Ro 03-8799 – pharmacokinetics, toxicology, tissue and tumor concentrations. Int J Radiat Oncol Biol Phys 10:1759–1763
45. Schwade JG, Strong JM, Gangji D (1980) I.V. misonidazole (NSC261037). Report of initial clinical experience. In: Radiation sensitizers: their use in the clinical management of cancer. Masson, USA, pp 414–420
46. Shibamoto Y, Takahashi M, Abe M (1996) A phase I study of a hypoxic cell sensitizer KU-2285 in combination with conventional radiotherapy. Radiother Oncol 40:55–58
47. Stratford MR, Minchinton AI, Dische S, Saunders MI, Anderson P (1982) Desmethylmisonidazole (Ro 05-9963): clinical pharmacokinetics after multiple oral administration. Int J Radiat Oncol Biol Phys 8:377–379
48. Thomas GM, Rauth AM, Bush RS, Black BE, Cummings BJ (1980) A toxicity study of daily dose metronidazole with pelvic irradiation. Cancer Clin Trials 3:223–230
49. Timothy AR, Overgaard J, Overgaard M (1984) A phase I clinical study of nimorazole as a hypoxic radiosensitizer. Int J Radiat Oncol Biol Phys 10:1765–1768
50. Urtasun RC, Chapman JD, Band P, Rabin H, Fryer CG, Sturmwind J (1975) Phase I study of high-dose metronidazole: a specific in vivo and in vitro radiosensitizer of hypoxic cells. Radiology 117:129–133
51. Urtasun RC, Chapman JD, Feldstein ML, Band PR, Rabin HE, Wilson AE, Marynowski B, Starreveld A, Shnitka T (1978) Peripheral neuropathy related to misonidazole incidence and pathology. Br J Cancer 37:271–275
52. Urtasun RC, Coleman CN, Wasserman TH, Phillips TL (1984) Clinical trials with hypoxic cell sensitizers: time to retrench or time to push forward? Int J Radiat Oncol Biol Phys 10:1691–1696
53. Varghese AJ, Whitmore GF (1980) Binding to cellular macromolecules as a possible mechanism for the cytotoxicity of misonidazole. Cancer Res 40:2165–2169
54. Walton MI, Bleehen NM, Workman P (1985) The reversible N-oxidation of the nitroimidazole radiosensitizer Ro 03-8799. Biochem Pharmacol 34:3939–3940
55. Wiebe LI, Stypinski D (1996) Pharmacokinetics of SPECT radiopharmaceuticals for imaging hypoxic tissues. Q J Nucl Med 40:270–284
56. Workman P (1980) Pharmacokinetics of hypoxic cell radiosensitizers. Cancer Clin Trials 3:237–251
57. Workman P, Stratford IJ (1993) The experimental development of bioreductive drugs and their role in cancer therapy. Cancer Metastasis Rev 12:73–82
58. Workman P, Walton MI (1990) Enzyme directed bioreductive drug development. In: Selective activation of drugs by redox processes (NATO series A 198). Plenum Press, New York, pp 173–191