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Bioreductive Metabolism of the Novel Fluorinated 2-Nitroimidazole Hypoxia Probe *N*-(2-Hydroxy-3,3,3-trifluoropropyl)-2-(2-nitroimidazolyl) Acetamide (SR-4554)

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ABSTRACT. The aim of this work was to study the metabolic characteristics of the novel fluorinated 2-nitroimidazole hypoxia probe *N*-(2-hydroxy-3,3,3-trifluoropropyl)-2-(2-nitroimidazolyl) acetamide (SR-4554). HPLC and ^{19}F NMR methods were employed to evaluate the rate of reductive metabolism of SR-4554 and the nature of the resulting metabolites, respectively. SR-4554 was enzymatically reduced by mouse liver microsomes (1.1 ± 0.1 nmol of SR-4554 reduced/min/mg protein), purified rat and human NADPH:cytochrome P450 reductase (17.8 ± 0.4 and 5.0 ± 0.5 nmol of SR-4554 reduced/min/mg protein, respectively), and SCCVII tumour homogenates (2.3 ± 0.3 nmol of SR-4554 reduced/min/g tumour) under nitrogen. NADPH:cytochrome P450 reductase was a major microsomal enzyme involved in the bioreduction of SR-4554 by liver microsomes. In a panel of murine and human tumour xenografts, cytochrome P450 reductase activities were found to be low and only varied by 3-fold between different tumour types, suggesting that enzyme activities within the tumours are unlikely to influence markedly *in vivo* reductive metabolism. Reduction of SR-4554 by mouse liver microsomes showed a characteristic oxygen dependence with a half-maximal inhibition of 0.48 ± 0.06%. Thus, the reductive metabolism of SR-4554 can be employed to detect the low oxygen tensions that occur within both murine and human tumours. Soluble, low molecular weight reductive metabolites of SR-4554 were identified by ^{19}F NMR. These metabolite peaks appeared (up to 0.12 ppm) downfield of the parent drug peak. In conclusion, SR-4554 undergoes an oxygen-dependent metabolism that involves NADPH:cytochrome P450 reductase. ^{19}F NMR is capable of identifying reduced metabolites that are undetectable by HPLC. BIOCHEM PHARMACOL **54**;11:1217–1224, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. hypoxia; SR-4554; reductive metabolism; NADPH:cytochrome P450 reductase; 2-nitroimidazoles; microsomes

2-Nitroimidazoles have been used extensively as radiosensitizers, chemosensitizers, and hypoxia-directed cytotoxins [1, 2]. More recently, 2-nitroimidazoles containing labels such as ^{123}I, ^{18}F, and ^{19}F have been developed as surgically non-invasive probes for detecting tumour hypoxia by single photon emission computed tomography (SPECT) [3, 4], positron emission tomography (PET) [5], and magnetic resonance spectroscopy (MRS) [6, 7]. The usefulness of these compounds as non-invasive probes is based on their ability to undergo nitroreduction and selective retention within hypoxic tumour cells [8–10].

A wide variety of enzymes including NADPH:cytochrome P450 reductase (EC 1.6.2.4), cytochrome P450, xanthine oxidase, DT-diaphorase, and aldehyde dehydrogenase can potentially act as nitroreductases, although many of these appear to have low substrate specificities for the reduction of 2-nitroimidazoles [11]. The major membrane-bound microsomal nitroreductases in liver and tumours are NADPH:cytochrome P450 reductase and cytochrome P450 [11–14]. For instance, using an HPLC assay, Walton and Workman [12] showed that the loss (reduction) of benznidazole in mouse liver preparations is dependent on NADPH:cytochrome P450 reductase levels, while formation of its amine metabolite is dependent on both NADPH:cytochrome P450 reductase and cytochrome P450 levels. Joseph *et al.* [13], on the other hand, transfected monkey kidney cells with recombinant plasmids in order to effect intracellular overexpression of NADPH:cytochrome P450 reductase and DT-diaphorase. An increase (5- to 7-fold) in metabolic binding of 2-nitroimidazoles was demonstrated in the NADPH:cytochrome P450 reductase cell line (80-fold increased activity), whereas the DT-diapho-

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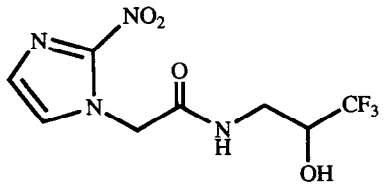

FIG. 1. Structural formula of SR-4554.

rase cell line showed only a small (1.5-fold) increase in metabolic binding of 2-nitroimidazoles, in spite of the increased activity (1000-fold) of DT-diaphorase in this cell line [13]. From their data, Joseph *et al.* [13] suggested that the capacity of a tissue to bind 2-nitroimidazoles under hypoxia should be proportional to the square root of its intracellular NADPH:cytochrome P450 reductase activity. Interestingly, both Walton and Workman [12] and Joseph *et al.* [13] demonstrated in *in vitro* experiments that xanthine oxidase is a relatively inefficient enzyme for 2-nitroimidazole reduction. Using purified buttermilk xanthine oxidase, however, Prekeges *et al.* [15] demonstrated the capability of this cytosolic nitroreductase to reduce the 2-nitroimidazole fluoromisonidazole.

Different experimental methods have been used to characterize the reductive metabolism of 2-nitroimidazoles. Radiochemical methods have been employed to measure levels of 2-nitroimidazole adducts in the acid-insoluble and acid-soluble fractions of incubation mixtures [13, 16]. HPLC with UV detection, as well as direct UV spectrophotometric methods, can measure either the removal of parent drug (loss of nitro chromophore) or the formation of the amine metabolites [12, 15]. Although loss of a parent drug and adduct or amine formation do not occur at similar rates, both processes occur as a function of enzyme levels and oxygen content and can be used to characterize reduction of 2-nitroimidazoles.

N-(2-Hydroxy-3,3,3-trifluoropropyl)-2-(2-nitroimidazolyl) acetamide (SR-4554) (Fig. 1) is a novel, non-invasive MRS probe for detecting hypoxia within tumours [17, 18]. The compound has been designed to have suitable metabolic and pharmacokinetic properties [19]. In A2780 spheroids, SR-4554 localizes predominantly within the hypoxic regions of the spheroids [17]. Subcellular localization studies demonstrated that within cells from the hypoxic region of A2780 spheroids, SR-4554 is associated mainly with the endoplasmic reticulum, nucleus, nuclear periphery, and cytoplasmic side of intracellular vesicles [17]. The studies suggested that bioreduced metabolites of SR-4554 are retained within those hypoxic cells in which reductive activation occurred. In this paper, the metabolism of SR-4554 has been characterized using mouse liver microsomes, purified reductase enzymes, and tumour homogenates. An HPLC method [20] has been employed to detect loss of parent drug through nitroreduction. In addition, a high resolution ^{19}F NMR technique has also been implemented to study metabolite formation. Finally, the activity of NADPH:cytochrome P450 reductase enzyme in a panel of murine tumour xenografts has been determined to ascertain the capability of these tumours to reduce 2-nitroimidazoles. The results of these studies are relevant to the interpretation of *in vivo* MRS studies where the oxygenation status of tumours is evaluated using SR-4554 [18].

MATERIALS AND METHODS

Chemicals

SR-4554 was synthesized and supplied by SRI International, Menlo Park, CA, U.S.A. [19]. The internal standard, 1-(2-nitro-1-imidazolyl)-3-chloro-2-propanol (Ro 07-0269), was synthesized and supplied by Roche Products, Welwyn Garden City, Herts, U.K. The compounds were assessed for chromatographic purity (98.5%) and used without further purification. Thallium chloride ($TlCl_3 \cdot 4H_2O$), cytochrome *c*, bovine albumin, and NADPH were obtained from the Sigma Chemical Co. (Dorset, U.K.). The Bio-Rad protein assay kit was obtained from BIO-RAD Laboratories (Hertfordshire, U.K.). Purified rat and human NADPH:cytochrome P450 reductase protein were obtained from Prof. C. R. Wolf (ICRF Laboratory of Molecular Pharmacology, Dundee, U.K.) [21, 22]. Zero grade N_2 gas (99.999%) and various gas mixtures of oxygen in N_2 were obtained from Air Products Ltd. (Glasgow, U.K.). All other reagents were analytical or HPLC grade.

Experimental Animals and Tumours

Mice were obtained from Harlan Olac Ltd. (Oxon, U.K.). They were allowed laboratory chow and water *ad lib.* and

TABLE 1. Sources and characteristics of cell lines

Cell Line	Mouse Strain	Histology	Source	Reference
RIF-1	C3H	Fibrosarcoma	St. George's Hospital Medical School, London, U.K.	[23]
SCCVII	C3H	Squamous cell carcinoma	MRC Radiobiology Unit, Didcot, U.K.	[24]
KHT	C3H	Fibrosarcoma	MRC Radiobiology Unit, Didcot, U.K.	[25]
EMT6	Balb/c	Adenocarcinoma	MRC Clinical Oncology Unit, Cambridge, U.K.	[26]

used when they were 6–8 weeks old. Cell lines (Table 1) were shown to be free from mycoplasma and were grown s.c. on the flank of the recipient mice. Tumours ranged between 200 and 500 mm^3 at the time of experiment. Liver microsomes were prepared from livers of non-tumour bearing male Balb/c mice.

Preparation of Liver and Tumour Samples

All tissues (livers and tumours) were excised rapidly, washed, and kept at 4° throughout the experiment to avoid loss of enzyme activity. Microsomes and S9 fractions were prepared by a modification of the method of Omura and Sato [27]. Tissues were weighed and homogenized in an equivalent volume of cold (4°) 50 mM Tris–150 mM KCl–HCl buffer (pH 7.4), using a glass–teflon homogenizer. The homogenates were centrifuged initially (10,000 × *g* for 30 min) at 4° to remove nuclei, mitochondria, and cell debris. The supernatant obtained from this initial step was the S9 fraction. To obtain liver microsomes, the liver S9 fraction was further centrifuged at 100,000 × *g* for 1 hr (at 4°). The pellet obtained by this process was resuspended and recentrifuged (100,000 × *g* for 1 hr) at 4°. The pellet obtained from this final centrifugation step and the S9 fractions were stored at −70° prior to use. The fractional yield of microsomes using this procedure was ~15%. Reconstituted microsomes and S9 fractions could be stored at −70° for up to 6 weeks without loss of activity.

Protein and Enzyme Assay

The protein concentrations of microsomes and S9 fractions were assayed by a Bio-Rad protein assay kit that measures the absorbance at 595 nm of an acidic solution of coomassie blue–protein complex [28]. Standard concentrations of bovine albumin were used to obtain a calibration curve.

Tissue NADPH:cytochrome P450 reductase activity was determined by measuring the reduction of cytochrome *c* at 550 nm [28]. Cytochrome P450 activity in liver microsomes was determined by difference spectroscopy as described by Omura and Sato [27, 29].

Metabolism Studies

The metabolism of SR-4554 was evaluated under hypoxia (N_2 gas; $O_2 < 0.001\%$) in an incubation mixture (complete system) composed of SR-4554 (50 μM), mouse liver microsomes (2 mg/mL), and excess cofactor (5 mM NADPH) in 100 mM Tris–HCl buffer (pH 7.4). Samples (3 mL) contained in specially adapted 25-mL conical flasks were maintained at 37° in a water bath with agitation at 150 oscillations/min. Each hypoxic incubation was preceded by preincubation of microsomes, cofactor, and buffer (0.1 M Tris–HCl, pH 7.4) with N_2 for 8 min. Inhibitory studies were carried out using (0.2 mg/mL) $TlCl_3 \cdot 4H_2O$ (an inhibitor of NADPH:cytochrome P450 reductase [30]), carbon monoxide (an inhibitor of cytochrome P450), and air (contains 21% O_2, which is an inhibitor of reductive metabolism) [12, 21].

To study the effect of microsomal protein concentration on the rate of metabolism of SR-4554 (50 μM), the compound was incubated with microsomal protein concentrations ranging between 0.2 and 8 mg/mL. Experiments designed to evaluate the specific relationship between O_2 concentration of the gas phase and the rate of microsomal metabolism of SR-4554 (50 μM) were performed as above (complete system), but in place of N_2 gas, different gas mixtures of O_2 (0, 0.01, 0.1, 0.2, 0.4, 0.7, 1, 5, and 21%) in N_2 were used. The rate of SR-4554 reduction versus percent O_2 in gas phase was modeled using GraphPAD Inplot Version 3.14 (GraphPAD Software Inc., San Diego, CA, U.S.A.).

The role of NADPH:cytochrome P450 reductase in SR-4554 metabolism was confirmed by evaluating the reduction of SR-4554 (50 μM) by both purified human and rat NADPH:cytochrome P450 reductase. The experiments were carried out as above, but in place of microsomal protein, 1.3 U/mL (1 U = 1 μmol cytochrome *c* reduced/min/mg protein measured at 37°) of purified human or rat NADPH:cytochrome P450 reductase and 5 mg/mL bovine albumin were used. In addition, this reaction was carried out with and without 0.2 mg/mL of $TlCl_3 \cdot 4H_2O$. The reduction of 50 μM SR-4554 by tumour homogenates under hypoxia was investigated. As a typical example, SCCVII tumours were used to reduce SR-4554. Aliquots of SCCVII whole tumour homogenates [50% (w/v) in 0.1 M Tris–HCl buffer, pH 7.4] were used as the enzyme source, similar to that described by Walton and Workman [12]. Specifically, 800 μL of the tumour homogenate was used per 3 mL of the incubation mixture. This reaction was also carried out in the presence of $TlCl_3 \cdot 4H_2O$.

Analysis of Samples by HPLC

Aliquots (250 μL) of the incubation mixture were withdrawn at 0, 3, 6, 9, 12, and 15 min into Eppendorf tubes containing the internal standard Ro 07-0269 (20 μL, 125 μg/mL). The incubation samples were extracted immediately with 30% (w/v) $AgNO_3$ solution (25 μL) and kept on ice prior to analysis. Aliquots (50 μL) of the extracts were directly analyzed by reversed-phase HPLC as previously described [20]. Briefly, separation of analytes was performed on a μ-Bondapak C_{18} (10 μm, 300 × 3.9 mm) analytical column (Millipore) at ambient temperature. The mobile phase consisted of methanol:water (15:85) delivered isocratically at a flow rate of 2 mL/min. The column effluent was monitored by UV-photodiode array detection at 324 nm, and the peak area ratio of SR-4554 to internal standard (Ro 07-0269) was used for conversion of the detector response to concentration estimates.

TABLE 2. NADPH:cytochrome P450 reductase activities in a panel of murine and human tumour xenografts

Tumour type	NADPH:cytochrome P450 reductase activity (nmol/min/mg)*
Murine	
EMT6	6.9 ± 0.4†
SCCVII	5.0 ± 0.7
RIF-1	4.2 ± 0.5
KHT	3.4 ± 0.4
Human	
HN5	11.9 ± 1.8
WIL	10.5 ± 1.2
BE	6.6 ± 1.3
HT-29	5.2 ± 0.2

* Units of activity: nmol of cytochrome c reduced/min/mg protein.
† Mean ± 1 SEM calculated from four individual tumours per tumour type.

Analysis of Samples by High Resolution ^{19}F NMR

High resolution NMR was used to evaluate the formation of SR-4554 metabolites. SR-4554 (250 μM) was incubated under hypoxia with 2 mg/mL mouse liver microsomes and excess cofactor (5 mM NADPH) as described above. Aliquots (0.7 mL) were withdrawn after 30 min of incubation, freeze dried, and reconstituted in 0.7 mL D_2O for NMR analysis. Samples were also extracted with 30% (w/v) $AgNO_3$ (1:10, v/v) or filtered through an ultrafiltration membrane with a cut-of of approximately 30,000 (Amicon Div., W. R. Grace & Co., Danvers, MA, U.S.A.) prior to freeze drying and analysis. High resolution ^{19}F NMR analysis was carried out on a Bruker AM 200 (Aspec 3000), 200 MHz NMR system using a total of 2048 scans and operating in ^{1}H decoupled mode at 25°.

Statistical Analysis

Statistical parameters were generated using either Minitab, Release 9 (Minitab, State College, PA, U.S.A.) or Microsoft Excel, Version 4.0 (Microsoft, Redmond, WA, U.S.A.).

RESULTS

Enzyme Activity

To compare the activity of NADPH:cytochrome P450 reductase in mouse liver and tumours, S9 fractions of these tissues were prepared and used to measure the reduction of cytochrome *c*. Reductase activity was high in liver microsomes and S9 [231 ± 6 and 121 ± 15 nmol of cytochrome *c* reduced/min/mg protein, respectively (mean ± SEM, N = 3)] but low in all tumours (Table 2). A 3-fold variation in enzyme activity was observed between these tumours. Since mouse liver microsomes were employed to characterize SR-4554 metabolism, the level of cytochrome P450 in the microsomes was also determined by difference spectroscopy and found to be 1.03 ± 0.06 nmol/mg (mean ± SEM, N = 3).

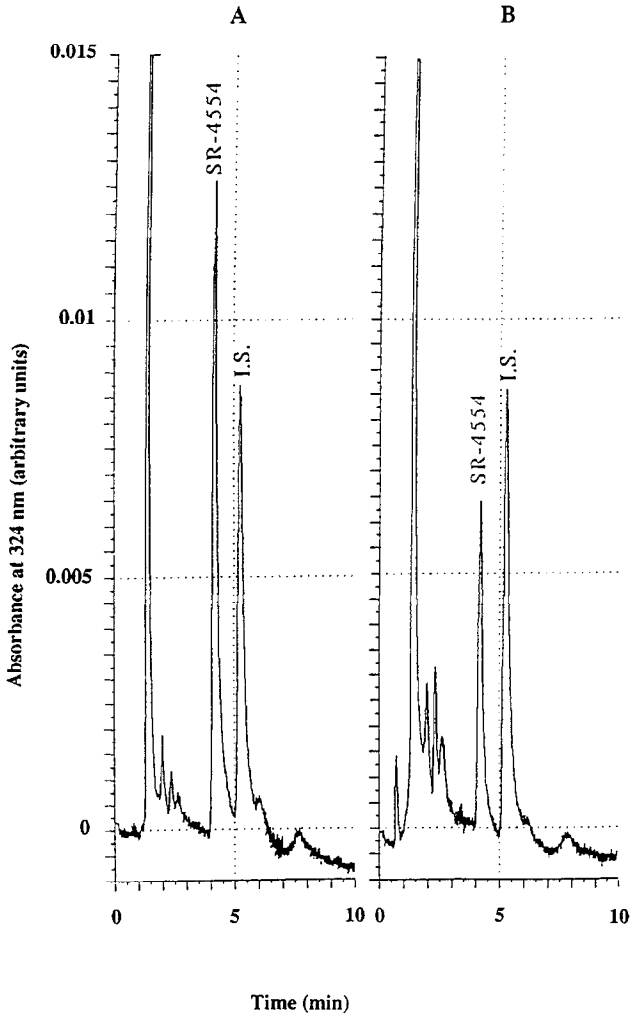

FIG. 2. Typical HPLC chromatograms of extracts obtained from the hypoxic incubation of SR-4554 at zero time (A) and 15 min (B). The chromatograms show the solvent front; SR-4554; and the internal standard (I.S.; Ro 07-0269), and also demonstrate parent drug loss at the latter time point. The minor peaks are contaminants from the microsome–NADPH mixture.

HPLC Analysis

SR-4554 was reduced by mouse liver microsomes under hypoxia. This was assessed by measuring the time dependence of parent drug loss, using HPLC. Figure 2 illustrates typical chromatograms of extracts obtained from the incubation of SR-4554 with mouse liver microsomes under hypoxic conditions. SR-4554 concentrations were calculated from peak area ratio of SR-4554 to internal standard (Ro 07-0269). The rate of metabolism was obtained from the slope of SR-4554 concentration versus time plots, which were linear within the time studied (15 min).

Characteristics of Reductive Metabolism

Figure 3 shows the characteristics of the reductive metabolism of SR-4554 by mouse liver microsomes. The reductive metabolism was inhibited completely in air, in the

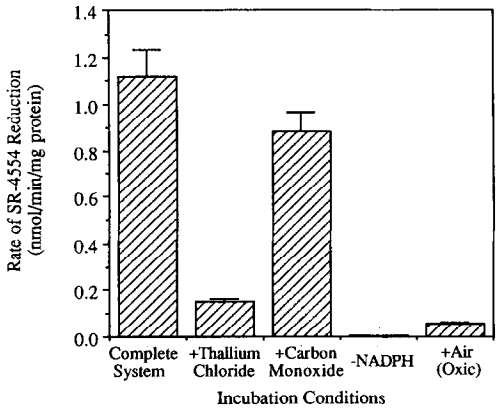

FIG. 3. Characteristics of the metabolism of SR-4554 by mouse liver microsomes. In the complete system, SR-4554 (50 μM) was metabolized in the presence of 2 mg/mL microsomal protein and 5 mM NADPH under hypoxia. The mean rate of reaction (nmol of SR-4554 reduced/min/mg protein) ± SEM (N = 3) is presented.

absence of cofactor (NADPH), and in the presence of $TlCl_3 \cdot 4H_2O$ (an inhibitor of NADPH:cytochrome P450 reductase). In contrast, however, carbon monoxide (an inhibitor of cytochrome P450) only caused a 21% inhibition of microsomal metabolism (Fig. 3).

The rate of metabolism of SR-4554 increased linearly ($r^2 = 0.99$) with an increase in microsomal protein concentrations up to the highest protein concentration (8 mg/mL) studied. Whereas no measurable reduction of (50 μM) SR-4554 was observed at protein concentrations below 0.5 mg/mL, complete reduction occurred at protein concentrations of 4 and 8 mg/mL within 15 min. Even under these latter conditions, no metabolites of SR-4554 were identified on the chromatogram.

To assess the potential of SR-4554 as a hypoxia probe and following the complete inhibition of its reduction by air (see above), the relationship between reduction of SR-4554 and oxygen content of incubation mixtures was investigated. The results of this study are presented in Fig. 4. SR-4554 metabolism was observed to vary markedly

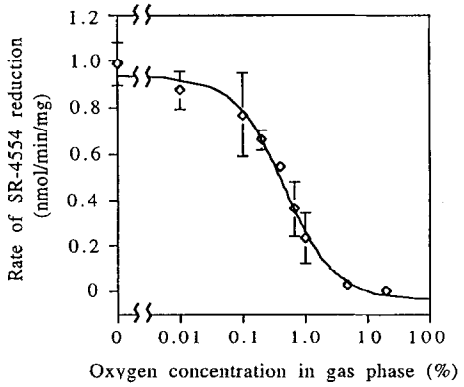

FIG. 4. Rate of reduction of SR-4554 (nmol SR-4554 reduced/min/mg protein) by mouse liver microsomes at various oxygen concentrations.

between 0.05 and 5%. The slope of the linear portion of the curve was -1.1 ± 0.17, and the concentration of oxygen required to decrease the hypoxia-dependent metabolism by 50% compared with nitrogen (half-maximal inhibition) was estimated to be $0.48 \pm 0.06\%$.

The characteristics of SR-4554 reduction by mouse liver microsomes (Fig. 3) suggested that NADPH:cytochrome P450 reductase was an important enzyme for this reaction. To confirm this observation, purified NADPH:cytochrome P450 reductase was used as the enzyme source to metabolize SR-4554. SR-4554 was, as expected, metabolized by both rat and human NADPH:cytochrome P450 reductase. Table 3 shows that the rat enzyme metabolized SR-4554 at a higher rate than the human enzyme, suggesting interspecies variation. The reduction of SR-4554 by both the rat and human enzymes was inhibited completely by $TlCl_3 \cdot 4H_2O$. Table 3 also demonstrates that SR-4554 was reduced by SCCVII tumour homogenates at measurable rates. This reaction was inhibited by 60% when $TlCl_3 \cdot 4H_2O$ was included in the incubation mixture.

Detection of Reductive Metabolites

The inability of the HPLC method used in this paper to detect SR-4554 metabolites, despite the loss of parent drug, was most likely due to loss of the UV chromophore following reduction of the nitro group. This prompted investigation into alternative analytical methods to detect reductive metabolites. In the absence of radiolabelled SR-4554, a ^{19}F NMR approach was used for this. An NMR method was also employed because SR-4554 is under development as a magnetic resonance probe for tumour hypoxia. *In vitro* studies with microsomes using ^{19}F NMR showed the presence of three SR-4554 metabolite peaks at 0.06, 0.07, and 0.12 ppm (10, 16, and 23 Hz, respectively) downfield from the parent drug peak (Fig. 5). The metabolite(s) giving rise to these peaks could not be assigned specifically to a particular compound or adduct. However, the same peaks were found to be present in the supernatant of silver nitrate precipitates and in ultrafiltrates obtained by passing incubation mixtures through an ultrafiltration membrane (cut-off 30,000), but not in the resuspended pellet of silver nitrate precipitates.

DISCUSSION

The metabolism of 2-nitroimidazoles has been shown to be dependent mainly on oxygen tension and enzyme content of tissues [12, 13, 16, 31]. In this paper, the characteristics of the reductive metabolism for a novel hypoxia probe, SR-4554, were investigated. Apart from its suitable pharmacokinetic profile [19], SR-4554 was also designed to have decreased susceptibility to side-chain oxidation reactions by the introduction of an amide function in the side chain. Such unwanted reactions occur with misonidazole and misonidazole analogues [10, 31–33], and make them less suitable as markers for tumour hypoxia.

TABLE 3. Reduction of SR-4554 by purified enzymes and tumour homogenates

Enzyme source	Rate of SR-4554 reduction*	
	Complete system†	$+TlCl_3 \cdot 4H_2O$‡
Purified rat NADPH:cytochrome P450 reductase	17.8 ± 0.4§	Not detected
Purified human NADPH:cytochrome P450 reductase	5.0 ± 0.5	Not detected
SCCVII tumour homogenate	2.3 ± 0.3	0.9 ± 0.1

* Units of rate: nmol of SR-4554 reduced/min/mg protein and nmol of SR-4554 reduced/min/g tumour for purified enzyme and tumour homogenate preparations, respectively.

† The complete system contained an enzyme source (1.3 U/mL of purified enzyme or 800 mL of SCCVII tumour homogenate (50%, w/v)/3 mL), 5 mM NADPH, and 50 mM SR-4554.

‡ Complete system with 0.2 mg/mL $TlCl_3 \cdot 4H_2O$, an inhibitor of cytochrome P450.

§ Data represent mean reduction rates ± SEM (purified enzymes, N = 4; tumour homogenate, N = 3).

The loss of parent drug, as determined by HPLC with UV detection, was used as a measure of SR-4554 nitroreduction. No metabolites were observed by this HPLC method, even at the highest microsomal protein concentration used. This may be due, in part, to the stability of the amide function, as well as the absence of a nitro chromophore (responsible for the characteristic UV absorption of SR-4554 at 325 nm) in reduced metabolites. Walton and Workman [12], however, detected the amine metabolite of the 2-nitroimidazole benznidazole using a similar technique. In this case, the additional chromophore introduced by the aromatic ring in the N-1 side chain of benznidazole was responsible for the UV detection capability at the lower λ_{max} of the amine metabolite [12]. In the majority of cases, however, reductive metabolism of 2-nitroimidazoles is characterized by formation of covalent adducts of reactive intermediates rather than the terminal six-electron reduced amine products [8, 9, 16, 34, 35].

The reductive metabolism of SR-4554 in mouse liver microsomes was found to be dependent on two enzymes, NADPH:cytochrome P450 reductase and cytochrome P450. Inhibitory studies with the NADPH:cytochrome P450 reductase inhibitor $TlCl_3 \cdot 4H_2O$ [12, 30] indicated that NADPH:cytochrome P450 reductase was the major enzyme in this reductive process. Carbon monoxide was a less efficient inhibitor of SR-4554 loss, suggesting that cytochrome P450 was not a major reductase for this compound. The importance of NADPH:cytochrome P450 reductase in the reductive metabolism of SR-4554 in mouse liver microsomes was confirmed by metabolism with purified rat and human NADPH:cytochrome P450 reductases. All murine and human tumour xenografts analyzed showed activity for NADPH:cytochrome P450 reductase in the range of 3.4 to 11.9 nmol cytochrome *c* reduced/min/mg protein. The activities in these tumours were found to be similar to those reported for untransformed cell lines [36] and biopsy samples of gliomas [14]. In general, the reductase activity in these tumours was 10- to 30-fold less than that in liver. The higher reductase activity in liver compared with tumours may account, at least in part, for its higher reductive capacity, e.g. for the 2-nitroimidazole benzidazole (12- to 50-fold) compared with mouse tumours such as EMT6, RIF-1, and KHT [12].

The ability of tumour homogenates to reductively activate SR-4554 at measurable rates was investigated by HPLC. This was particularly important considering the inherent insensitivity of magnetic resonance techniques. SR-4554, at achievable tumour concentrations of 50 μM [19], was reduced by SCCVII at measurable rates (2.3 ± 0.3 nmol of SR-4554 reduced/min/mg tumour). This will translate into ~70% reduction of 50 μM SR-4554 by 1 g of tumour within 15 min under hypoxia (N_2) and in the presence of adequate NADPH. The possibility that reductases other than NADPH:cytochrome P450 reductase may be involved in the tumour metabolism of the compound was suggested by experiments with the NADPH:cytochrome P450 reductase inhibitor $TlCl_3 \cdot 4H_2O$, which showed only 60% inhibition of reductive metabolism.

Importantly, SR-4554 metabolism was shown to be markedly dependent on oxygen tension. The reductive process was inhibited completely in air (21% oxygen) and partially inhibited by increasing levels of oxygen from 0.01 to 5%. The oxygen concentration that produced 50% inhibition of SR-4554 reduction between air and nitrogen (0.48%) was similar to that previously reported for the formation of covalent adducts by radiolabelled 2-nitroimidazoles such as misonidazole and desmethylmisonidazole [10, 13, 31]. This observation is interesting, since the effect of varying oxygen tensions on the *in vitro* reduction rates of 2-nitroimidazoles (i.e. loss of parent drug) *per se* has not been reported previously. The enzyme-profiling studies demonstrated that NADPH:cytochrome P450 reductase activity does not vary markedly in the panel of tumours studied. Considering the square root relationship between enzyme activity and 2-nitroimidazole activation [13], the studies suggest that enzyme activity will influence reductive metabolism of 2-nitroimidazoles within these tumours to a lesser extent than does the effect of oxygen.

Although no metabolites of SR-4554 were detectable by HPLC, these were seen in high resolution ^{19}F NMR studies. Hypoxic reduction of SR-4554 was characterized by three metabolite peaks in microsomal incubation mixtures. The chemical shift differences (up to 0.12 ppm) were, however, too small to give resolved peaks in *in vivo* MRS studies. Other fluorinated 2-nitroimidazoles, such as fluoromisonidazole (Ro 07-0741) and trifluoroethylmisonidazole

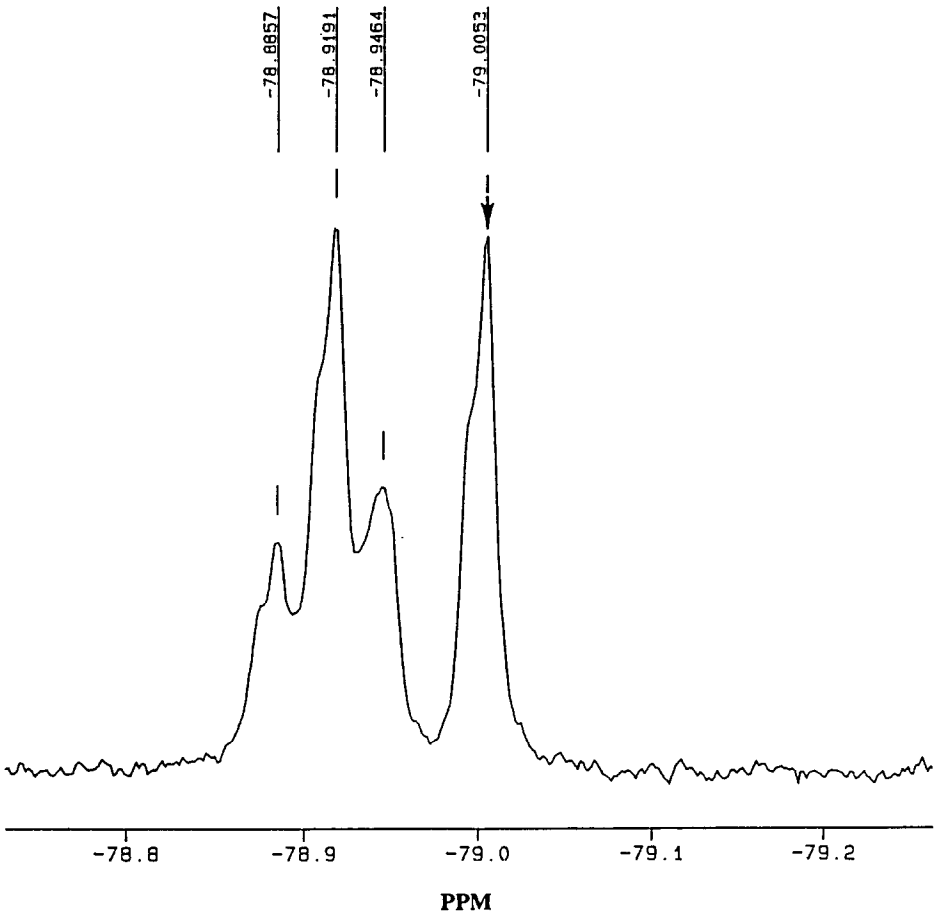

FIG. 5. Typical high resolution NMR spectrum of a microsomal extract, obtained 30 min after the incubation of SR-4554 under hypoxia. The spectrum represents original SR-4554 (vertical arrow) and its hypoxic metabolites. The formation of these metabolites was inhibited when incubations were carried out in air.

(Ro 07-2044), have also been reported to undergo reductive metabolism to metabolites, which are chemically shifted downfield of the parent compounds [37]. The reductive metabolites of SR-4554 were found to have low molecular weight (less than that of plasma proteins; 30,000). The exact nature of these low molecular weight metabolites could not be fully elucidated. It is worth noting that the higher proportion of 2-nitroimidazole adducts are represented by those bound to RNA and glutathione, both of which are low molecular weight compounds [9, 32]. Future studies will attempt to evaluate whether some of these candidate adducts contribute to the observed NMR metabolite peaks. The ^{19}F NMR technique will, therefore, complement previous methods that used radiolabeled 2-nitroimidazoles to study the formation of covalent adducts in acid-insoluble and acid-soluble fractions of tumour homogenates, cancer cells, and microsomal preparations [8, 9, 16, 32, 28].

In conclusion, the novel 2-nitroimidazole SR-4554 undergoes hypoxia- or oxygen-dependent metabolism. This process involves NADPH:cytochrome P450 reductase and is inhibited by increasing levels of oxygen. Soluble reductive metabolites of SR-4554 were detected by ^{19}F NMR. These findings are relevant to the development of SR-4554 as a probe for tumour hypoxia.

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