The novel fluorinated 2-nitroimidazole hypoxia probe SR-4554: reductive metabolism and semiquantitative localisation in human ovarian cancer multicellular spheroids as measured by electron energy loss spectroscopic analysis

EO Aboagye1, AD Lewis1, A Johnson2, P Workman1,3, M Tracy4 and IM Huxham2

1CRC Department of Medical Oncology, University of Glasgow, Beatson Laboratories, Switchback Road, Glasgow G61 1BD, UK; 2Division of Molecular and Cellular Biology, University of Glasgow, Glasgow G12 8QQ, UK; 3Present address: ZENECA Pharmaceuticals, Cancer Research Department, Macclesfield SK10 4TG, UK; 4Life Sciences Division, SRI International, Menlo Park, California 94025, USA.

Summary The novel fluorinated 2-nitroimidazole SR-4554 is undergoing preclinical development as a magnetic resonance spectroscopy and imaging probe for hypoxic tumour cells. We have used electron energy loss spectroscopic analysis (EELS) to show selective reduction and differential subcellular localisation of SR-4554 in human ovarian multicellular spheroids. SR-4554 was demonstrated to be metabolised by these A2780 cells under hypoxic but not under normal aerobic cell culture conditions. The EELS technique illustrated that the relative amount of drug within the cytoplasm of cells from both the inner region (150–160 μm from edge) and outer edge of the spheroid did not differ significantly after an initial 3 h incubation with drug. In contrast, an 8-fold differential between the amount of drug retained in the cytoplasm (primarily ribosomes and endoplasmic reticulum) of cells from the inner 1/3 outer regions of the spheroids was observed following a subsequent 2 h ‘chase’ culture in drug-free medium. Within cells from the hypoxic region of the spheroid, SR-4554 was mainly associated with the endoplasmic reticulum, nucleus and the cytoplasmic side of intracellular vesicles and also to a lesser extent with the nuclear periphery. Interestingly, the drug was only weakly associated with the mitochondria and plasma membrane of the cells. The characteristics of cellular and subcellular distribution of SR-4554 are consistent with the hypothesis that 2-nitroimidazole compounds undergo hypoxia-mediated enzymatic reduction to reactive species. These reactive species are retained in the cells in which they are metabolised through covalent association with subcellular components. These findings provide additional support for the clinical development of the drug as a non-invasive probe for tumour hypoxia and at the same time illustrate the utility of the EELS technique for examining the heterogeneity of drug distribution both between and within cells.

Keywords: electron energy loss spectroscopy; fluorinated 2-nitroimidazole; hypoxia probe

Human and animal tumours have been reported to contain regions of low oxygen tension or hypoxia (Thomlinson and Gray, 1955; Moulder and Rockwell, 1984; Rampling et al., 1994). Tumour cells existing under hypoxia may be more resistant to therapy for a variety of reasons. These factors include: the requirement for molecular oxygen in the fixation of radiation-induced radicals and drug-induced damage, and certain drug activation processes; non-cycling cell kinetics; and decreased drug uptake (Workman, 1991). In recent years strategies have been developed to identify hypoxic cells within tumours, in order to facilitate the rational selection of appropriate therapeutic regimens. In this regard, both surgically invasive and non-invasive techniques such as autoradiography, immunohistochemistry, magnetic resonance spectroscopy (MRS) and single positron emission tomography (SPECT) have been used to provide a clinically relevant approach to identifying these hypoxic cells within tumours (Chapman, 1984; Maxwell et al., 1988; Koh et al., 1991; Kwok et al., 1992; Lord et al., 1993; Hodgkiss et al., 1994; Raleigh et al., 1994).

Nitroimidazoles, including misonidazole, have been under evaluation as potential diagnostic probes for hypoxic cells. This is because of the specific reductive metabolism of the drugs to reactive metabolites which bind to macromolecules within hypoxic cells (Miller et al., 1982; Chapman et al., 1983). The characteristics of binding have been evaluated in various cell lines and tumours, although the exact nature of the metabolite is not known (Miller et al., 1982; Chapman et al., 1983). Interestingly, previous subcellular fractionation studies in EMT6 cells with [14C]misonidazole have indicated that 77% and 23% of the total activity is associated with the acid-insoluble (bound) and the acid-soluble fractions (unbound) respectively (Miller et al., 1982). Further, the acid-insoluble fraction is distributed among RNA (17%), DNA (1%), lipid (4%) and protein (1%) (Miller et al., 1982). In another study by the Edmonton group, the involvement of intracellular enzymes in the activation process was implicated, since the temperature dependence of this process showed an activation energy of 33.5 kcal mol⁻¹ (Chapman et al., 1983). This study also demonstrated that the binding rate of misonidazole within hypoxic cells was at least 50 times greater than within aerobic cells, and suggested that the activation sites may be lipid associated. The higher rate of binding and longer half-life of the bound metabolite compared with the parent unmetabolised compound are considered as favourable characteristics with regard to the use of 2-nitroimidazoles as possible markers for hypoxic cells.

Electron energy loss spectroscopic analysis (EELS) by electron spectroscopic imaging (ESI) is an emerging technique for the in situ examination of objects and compounds in cells and tissues, but has only recently been applied to an analysis of cancer therapeutic compounds (Huxham et al., 1992, 1993). Briefly, the technique relies upon measurement of the electron energy distribution of transmitted electrons that have lost energy following interaction with a specimen. This is done by recording an energy spectrum as a sequence of images with high spatial resolution. These filtered electron image sequences contain information about the elementspecific energy loss population (equivalent to energy loss edge) superimposed on the non-specific energy loss population (equivalent to background). Data are processed mathematically to obtain edge intensity information which relates directly to the elemental concentration of the specimen. Com-
paring the intensity of two elements, as performed in our study, can thus disclose semiquantitative elemental information on a local (nm) scale.

In the present investigation, we used energy electron loss spectroscopy to study the localisation of a novel fluorinated 2-nitroimidazole SR-4554 (Figure 1) within A2780 human ovarian multicellular spheroids. The A2780 spheroid model was chosen because it provides the coexistence of both aerobic and hypoxic cells under normal aerobic cell culture conditions, allowing manipulations on both types of cells to be carried out simultaneously. Moreover, we have previously used this spheroid model with EELS (Huxham et al., 1992, 1993) to assess the localisation of fluorene-containing drug bound to macromolecules within different cell populations and subcellular compartments. SR-4554 was designed to contain three equivalent fluorines within a metabolically stable side-chain of appropriate lipophilicity, while retaining a similar reduction potential to misonidazole. Currently, SR-4554 is undergoing preclinical evaluation as a non-invasive probe for tumour hypoxia by fluorine-based magnetic resonance spectroscopy (MRS) and imaging (MRI). Together with bioreductive metabolism studies which are reported here, the present localisation studies will help in our understanding of the characteristics of metabolism-induced binding of SR-4554 and of 2-nitroimidazoles in general.

Materials and methods

Metabolism studies

SR-4554 [N-(2-hydroxy-3,3,3 trifluoropropyl)-2-(2-nitro-1-imidazolyl) acetamide] was synthesised and supplied by SRI International, Menlo Park, CA, USA. A2780 cells were cultured as monolayers in RPMI medium (Life Technology, Paisley UK) supplemented with 10% (v/v) fetal calf serum (Globepharm, Esher, Sussex, UK), and 0.001% (v/v) insulin (Lewes, Sussex, UK). The cells were grown to near confluence, trypsinised and plated at a concentration of 5 × 10^5 cells ml^{-1} in 5 ml. Cells were allowed to adhere to the flask and the medium replaced with one containing 10 and 20 μM SR-4554. The flasks were then incubated for various lengths of time up to 3 h under hypoxic (98% nitrogen and 2% carbon dioxide) or aerobic (2% carbon dioxide and 20% oxygen in nitrogen) conditions. Drug-containing media obtained at 0, 0.5, 1, 2, 3 h were then analysed by high-performance liquid chromatography (HPLC).

HPLC analysis and rate of SR-4554 reduction

Aliquots (250 µl) of the incubation media were spiked with 20 µl of an internal standard (8 μg ml^{-1} Ro 07-0269 [1-(2-nitro-1-imidazolyl)-3-chloro-2-propanol] supplied by Roche, Welwyn Garden City, Herts, UK) and the mixture extracted with 25 µl of silver nitrate solution (30%, w/v). Samples were vortexed, centrifuged (at 1000 g) for 10 min, and the supernatants analysed by HPLC (Millipore UK Ltd, Watford, UK) using a C18 Bondapak analytical column and a mobile phase consisting of 15% methanol and water. The analytes were eluted at a flow rate of 2 ml min^{-1}, and the column effluent monitored at a wavelength of 324 nm. Calibration standards (0.35–35.46 μM) were prepared in RPMI medium and analysed under identical conditions to that above. The concentrations of parent drug in the incubation samples were determined and plotted against time. Rates of reduction were estimated from the initial slope of the concentration of SR-4554 vs time curve, which was linear within the time period studied.

Spheroid culture and EELS analysis

Human ovarian A2780 cells were plated (in RPMI medium) at a concentration of 2 × 10^5 cells 50 ml^{-1} and incubated in stirrer flasks at 37°C to form spheroids. After 3.5 days in culture, spheroids of approximately 0.8–1.4 mm in diameter were obtained for the EELS experiment. Initially the spheroids formed as aggregates, but after 3.5 days in culture they formed tight proliferating spheroids. Under these conditions the large spheroids develop a region of hypoxia between the outer cells and the necrotic core. For the present experiments, the outer most cell layer of the spheroid was taken to be aerobic, whereas cells in the inner region of the spheroid, at approximately 150–160 μm from the surface, but separate from any necrotic core, were selected as hypoxic as shown by Miller et al. (1989) using EM/TEM. A2780 spheroids are similar to EM/TEM spheroids in that both form tight spheroid structures. In this regard, spheroid models showing differences in oxygen levels and binding of 2-nitroimidazoles have previously been established (Franko et al., 1987; Franko et al., 1992).

The spheroids were incubated with culture medium containing a non-toxic concentration of 1 mM SR-4554 at 37°C for 3 h. Incubations were carried out under normal aerobic cell culture conditions. Half of the spheroids were then transferred onto ice to stop further reaction. The other half were washed with fresh culture medium and 'chased' at 37°C for 2 h under normal aerobic conditions. These were also transferred onto ice to stop further reaction.

For analytical electron microscopy, all spheroids were briefly washed in phosphate-buffered saline (PBS), and chemically fixed (on ice) with 1% glutaraldehyde in PBS for 1.5 h. The spheroids were dehydrated in a series of alcohols for embedding at low temperature in Lowicryl K4M, a nitrogen-free hydrophilic methacrylate resin, without the use of heavy metal stains to avoid electron scattering. Ultrathin sections were mounted onto 700 mesh copper grids for electron spectroscopic analysis using a Zeiss TEM902 microscope (Karl Zeiss Oberkochen, Oberkochen, Germany) operating at 80 kV and 12,000 × magnification. The microscope was fitted with an electron energy filter for analysis of the local electron energy associated with fluorine (K-edge onset at ΔE = 688 eV) and nitrogen (K-edge onset at ΔE = 405 eV). This novel technique has a resolution of about 2 nm and an energy resolution of about 5 eV (Huxham et al., 1992; Johnson et al., 1995). Energy-filtered sections of resin-embedded cells, and a theoretical detection limit for fluorine of 90 μm, were used.

Energy-filtered image sequences were recorded between ΔE = 650 and 750 eV for fluorine and between ΔE = 350 and 450 eV for nitrogen, changing only the (measurable) video-camera (Dage SIT, Michigan, MN, USA) kV setting between each sequence pair to accommodate the change in energy loss intensity relative to the dynamic range of the video-camera. Comparisons between defined regions within peripheral (outer) cells and cells approximately 150–160 μm from the edge of the spheroid (inner cells) were made using in-house software.

Energy loss contributions of both fluorine and nitrogen were measured following background modelling for each pixel in median filtered energy loss image sequence. This was accomplished using the least mean square determination of the parameters which describe the energy loss curve (deBrujin et al., 1993; Johnson et al., 1995). Cumulative background-stripped grey level values from 12 image sequences over a 30

Figure 1 Drug structure of SR-4554.
eV portion of the post-ionisation edge for fluorine and nitrogen were used to calculate elemental ratios. In this way, variations in the density of cellular material between one domain and another could be normalised as a function of the nitrogen content for each region of interest, to produce local semiquantitative elemental maps. Data were also expressed as average grey level value representing energy loss for fluorine as a function of section area for each pixel.

**Results**

**Metabolism of SR-4554 by human ovarian carcinoma cells**

SR-4554 was reduced by A2780 cells in culture under hypoxic conditions. This was assessed by chromatographic analysis of loss of parent drug metabolised by the cells in culture. Figure 2 shows a typical chromatogram of a hypoxic compared with an aerobic incubation sample also containing the internal standard (Ro 07-0269). No metabolites were observed. The rates of reduction of SR-4554 by A2780 cells under aerobic and hypoxic culture conditions are shown in Table I. These data, obtained only at 37°C, demonstrated the selective reduction of SR-4554 under hypoxia in contrast to normal aerobic conditions. As expected, the reduction rates increased with increasing substrate concentration.

**Localisation of SR-4554 within human ovarian carcinoma cells by EELS**

EELS analysis of spheroids incubated with SR-4554 enabled the localisation of the fluorine atoms in subcellular components as well as across different regions within the spheroid. Figure 3 shows an unstained, energy filtered image (reversed contrast) of A2780 cells from a spheroid after culture in SR-4554-containing medium, recorded at $\Delta$E = 150 eV. By means of spectroscopic imaging, most intracellular structures of unstained material, otherwise difficult to see without filtering, could be identified for analysis. Using our in-house image analysis software, regions of interest (ROI) for each electron spectroscopic image sequence (ESIS) were defined simply by drawing on the

![Figure 2](image-url)  
**Figure 2** Typical HPLC plot of SR-4554 after incubation with A2780 cells for 30 min under (a) aerobic and (b) hypoxic conditions. The conditions of HPLC were as described in the Materials and methods section. From left to right the three peaks illustrated indicate the solvent front, SR-4554 and internal standard (IS; Ro 07-0269) respectively.

![Figure 3](image-url)  
**Figure 3** Energy-filtered image of a portion of a heavy metal-free A2780 cell within SR-4554-treated multicellular spheroids after a 'chase' culture, recorded at $\Delta$E = 150eV, showing enhanced contrast in phosphorus-rich regions. The image shows a well-defined nuclear region (N), nuclear membrane (arrow), mitochondria (M) and a cytoplasmic region rich in polyribosomes (PR). Bar = 600 nm.

| Conditions | Drug concentration ($\mu$m) | Rate of loss (nmol h$^{-1}$ 10$^{-6}$ cells) |
|------------|---------------------------|---------------------------------------------|
| Aerobic    | 20                        | No loss detected                            |
|            | 10                        | No loss detected                            |
| Hypoxic    | 20                        | 7.54 ± 0.10                                 |
|            | 10                        | 4.32 ± 0.25                                 |

SR-4554 was incubated with A2780 cells under aerobic and hypoxic conditions. The levels of parent drug remaining at various time periods were determined as described in the Materials and methods section. The values for rate of loss of compound are means ± s.d. from at least three separate determinations.
reference image on the display screen. The same ROI was used for analysis of both nitrogen and fluorine ESIS sequences. The energy losses for nitrogen and fluorine were then determined by plotting the cumulative grey level value for the ROI for each ESIS. A representative projected distribution of fluorine (green) within a section of the inner region of the spheroid after the 'chase' process is shown in Figure 4. This represents residual bound drug within the cell.

An analysis of cytoplasmic domains (which includes cytoplasm, mitochondria, vesicle periphery and plasma membrane regions) of cells within A2780 spheroids (Figure 5) suggest that the drug was distributed into cells across the whole spheroid after a 3 h culture. Following chemical fixation of these spheroids, only marginally less drug was bound to intracellular components within cells of the inner region of the spheroid as compared with the equivalent components of cells in the outer region. More importantly, an 8-fold higher level of drug was present within the cytoplasmic domains of these inner cells after 'chasing' with drug-free media, as most of the drug which was present in the outer cells had diffused

![Figure 4](image-url)  
(a) Reference image of a section from an inner A2780 cell within SR-4554-treated multicellular spheroids after a 'chase' culture, recorded ΔE = 150eV. The image shows the plasma membrane (PM), vesicles (V), cytoplasm (C); nuclear membrane (NM), nucleus (N) and nucleolus (Nclo). (b) The same image upon which the projected fluorine distribution is superimposed (green). The green binary fluorine map simply shows regions in which fluorine was found to be present (i.e. a stripped grey level value above background). TIFF images were reproduced on a Kodak Colourerse printer (field width = 5µm).
away following the ‘chase’ process. This selective retention of drug by the inner cells is indicative of increased bioactivation within the inner cells of the spheroids.

Table II demonstrates that the drug distribution present within the cells from both the inner and outer regions of ‘chased’ spheroids was not uniform. Relative to the intrinsic nitrogen content of specific intracellular regions, which takes into account local variations in biological material, the amount of drug within the inner cells was found to be comparatively high at the periphery of intracellular vesicles, within the cytoplasm, in the nucleus and at its periphery. In contrast, the amount of drug was comparatively low within mitochondria and at the plasma membrane. Although the nitrogen atoms in the drug will contribute to the total nitrogen intensity signal of the EELS technique, by far the greater contribution will come from the biological macromolecules of the cell to which the drug is localised. When simply expressed in terms of the average amount of fluorescein per unit area of cell section, the data suggested that there was a relative accumulation of fluorescein at the nuclear periphery and in the nucleus compared with other regions, more representative of the projected fluorescein distribution shown in Figure 4. In contrast, and as expected, across all domains there was less drug (3 to 10-fold) localised within the outer cells than in the inner cells.

Table II The relative average fluorescein and fluorescein/nitrogen elemental ratios for six intracellular domains of both outer and inner cells from A2780 multicellular spheroids following culture with SR-4554.

| Domain | Nucleus | Nuclear periphery | Cytoplasm | Mitochondria | Vesicle periphery | Plasma membrane |
|--------|---------|-------------------|-----------|--------------|------------------|-----------------|
| F/N ratio |         |                   |           |              |                  |                 |
| Outer  | 0.03 ± 0.02  | 0.03 ± 0.01       | 0.11 ± 0.04 | ND*          | 0.07 ± 0.03      | ND              |
| Inner  | 0.21 ± 0.02  | 0.25 ± 0.08       | 0.29 ± 0.08 | 0.06 ± 0.02  | 0.31 ± 0.06      | 0.16 ± 0.05     |
| F/pixel |         |                   |           |              |                  |                 |
| Outer  | 0.65 ± 0.28  | 0.45 ± 0.19       | 0.75 ± 0.22 | ND           | 0.61 ± 0.18      | ND              |
| Inner  | 2.15 ± 0.55  | 4.25 ± 0.75       | 1.97 ± 0.46 | 0.85 ± 0.22  | 0.25 ± 0.06      | 0.49 ± 0.13     |

*Average value from 12 areas ± s.d. for each domain expressed as the relative amount of fluorescein per pixel (8 nm²) (F/pixel) or as a fluorescein to nitrogen ratio (F/N ratio) in arbitrary units.

Discussion

The localisation of 2-nitroimidazoles following reduction by tumour cells is important in our understanding of the use of these compounds as hypoxic probes. The human ovarian A2780 cell line was used in this study since this cell line has previously been shown to express cytochrome P450 reductase, an enzyme involved in the reductive metabolism of 2-nitroimidazoles, by enzyme assay and PCR analysis (data not shown). This cell line was also considered likely to develop a hypoxic region in spheroids over the size range used.

The characteristics of the metabolism-induced binding of 2-nitroimidazoles to macromolecules in hypoxic tissue are still not fully understood. In particular, the nature of the drug adducts and the subcellular localisation of these compounds has been poorly addressed in the literature to date. This paper addresses some of these issues with respect to the use of a novel fluorinated 2-nitroimidazole (SR-4554) in A2780 cells/spheroids and the application of the EELS technique. However, these results are also relevant to the action of other 2-nitroimidazoles. The structure of SR-4554 is based on that of etanidazole (Brown and Workman, 1980), while its lipophilicity is more similar to misonidazole. The lipophilicities for SR-4554, etanidazole and misonidazole being 0.634, 0.046, and 0.430 respectively. Interestingly, the distribution properties demonstrated in our study are more appropriate in terms of those of etanidazole-like oxygen-dependent binding of the compound rather than misonidazole (Workman, 1982; Franko et al., 1987; Kocha et al., 1993). As expected, SR-4554 was metabolised (reduced) selectively by hypoxic A2780 cells but not under aerobic conditions.

The use of the novel EELS technique permitted the localisation of the compound within human ovarian carcinoma spheroids. In cultures not subjected to ‘chasing’, no significant differential was observed between cells of the inner and outer regions of the spheroid. This presumably is due to the presence of both original parent drug and bound drug metabolites. Since the EELS technique maps only atoms such as fluorine, nitrogen and phosphorus it cannot distinguish between original parent and bound drug. The method of ‘chasing’ in drug-free media to differentiate between bound and unbound drug was, therefore, employed to allow the assessment and localisation of bound metabolites. Interestingly, the results we obtained in our study using spheroids and EELS were in accordance with metabolism-induced binding of 2-nitroimidazoles to macromolecules as previously demonstrated by other methods with limited spatial resolution such as autoradiography and immunohistochemistry (Miller et al., 1982; Lord et al., 1993). In contrast to these other methods, the EELS technique also has the ability to measure drug binding relation to the density of other local macromolecular components. Although some non-specific binding occurred in the cells of the outer (aerobic) region of
the spheroids, levels of fluorne in the cytoplasm of cells from
the inner (hypoxic) regions were 8-fold higher. This effect is
the more likely to occur in vivo, where elimation processes
will result in the removal of unbound drug compared with
the bound metabolite. Importantly, the localisation charac-
teristics shown in our study are very relevant to the use of
this novel fluorinated compound as a non-invasive marker of
tumour hypoxia in the clinic. The presence of these
magnetically equivalent fluorne atoms in the structure of the
compound, which remains intact on enzyme reduction, is
important in terms of its detection by resonance techniques
as well as by EELS.

Further studies using EELS enabled us to analyse in more
detail the subcellular localisation of the compound within
cells of both the outer and inner region of the spheroid. In
our study, the mitochondria and plasma membrane did not
appear to bind significant amounts of drug. Of interest,
however, is the high levels of the compound localised to
the nuclei periphery, nucleus and cytoplasm within the inner
cells. These data are in agreement with previous published
studies using subcellular fractionation of cells labelled with
[14C]mitosanezole (Miller et al., 1982), considering that the
molecular components in these regions consist mainly of
RNA, DNA, lipids and proteins. In addition, the distribu-
tion of 2-nitroimidazole to cytoplasm, nuclear and perinuclear
regions was also mentioned by Cline et al. (1994), who used
antibodies against CCI-103F/CCI-103F adducts to follow the
distribution of hypoxia and/or inducive enzymes within cells
of canine tumours. In contrast, and as expected, less drug
was distributed within all the domains in the outer cells.

The initial steps in the reductive metabolism of 2-
mitoimizoles are mainly catalysed by cytochrome P450
reductase and to a lesser extent cytochrome P450, both of
which are found in the endoplasmic reticulum (McCusman et
al., 1982; Walton and Workman, 1987). The subcellular
distribution of the SR-4554 compound demonstrated in our
study suggests that the reactive intermediate is short-lived
and binds to macromolecules within the vicinity of the
metabolism site or to nucleoholes such as RNA close to
these sites. Importantly, the evidence would suggest that
the reactive metabolites do not appear to migrate out of the
cells. This characteristic is also relevant to the design of bioreduc-
tive drugs, based on 2-nitroimidazoles, which will target the
nucleus of the cell to deliver radioisotopes or alkylating
moieties.

Currently, SR-4554 is undergoing preclinical development
before scheduled clinical trials as a probe for investigating
tumour hypoxia by non-invasive MRS. Important to the
direction of these studies, this paper describes the intracel-
ular distribution of the compound within hypoxic regions of
human ovarian carcinoma spheroids and is useful in the
interpretation of data from MRS studies. In addition, how-
ever, the EELS technique itself can also be used to study the
distribution of hypoxic regions within tumours labelled in
vivo with SR-4554, even though this will involve invasive
(biopsy) procedures. As a semiquantitative technique,
moreover, it offers the potential of measuring hypoxia on a
cell-to-cell basis at a molecular level with good resolution
compared with antibody techniques and also the investiga-
tion of drug to macromolecule interactions without the use of
radioisotopes.

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