Intracellular Localization of the Radiation Enhancer Motexafin Gadolinium Using Interferometric Fourier Fluorescence Microscopy

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ABSTRACT
Motexafin gadolinium (MGd) is a unique therapeutic agent that localizes in cancer cells and increases tumor response to ionizing radiation and certain chemotherapeutics. The in vitro intracellular localization, accumulation, and retention of MGd in murine EMT6 mammary sarcoma and Rif-1 fibrosarcoma cell lines were studied using interferometric Fourier fluorescence microscopy. MGd cellular uptake was semiquantified using its characteristic fluorescence emission band centered at 758 nm. Colocalization studies were performed using mitochondrial, endoplasmic reticulum, Golgi apparatus, nuclear, and lysosomal fluorescent organelle probes, and verified using interferometric Fourier spectroscopy. Cellular uptake was gradual and increased significantly with incubation time. MGd localized primarily within the lysosomes and endoplasmic reticulum, and to a lesser extent within the Golgi apparatus and mitochondria. Mitochondrial staining was increased in media without serum. No nuclear uptake was detected in the Rif-1 cells, but after 48 h nuclear uptake was observed in 15% of EMT6 cells. These results indicated that MGd accumulates within cytoplasmic compartments. The sustained intracellular localization of MGd may, in part, account for its unique radiation and chemotherapy enhancement properties. Interferometric Fourier fluorescence microscopy is a potentially powerful tool in delineating and verifying localization sites of therapeutic agents.

Fluorescence microscopy enables the spatial assignment of fluorophores within cells. The limitations of current fluorescence cellular imaging, which mainly uses filter-based systems, include endogenous autofluorescence, exogenous dye contributions, and spectral overlap between probes and the studied fluorophore. Autofluorescence artifacts occur during biological imaging; these may distort experimental findings. The major endogenous fluorophores responsible for native fluorescence include aromatic amino acids (tryptophan, 340 nm), pyridoxine (390 nm), collagen (390 nm), elastin (410 nm), reduced nicotinamide adenine dinucleotides (470 nm), flavins (550 nm), and porphyrins (630 and 690 nm), all of which may perturb single point or filter detection systems (Richards-Kortum and Sevick-Muraca, 1996). Exogenous fluorophores also may interfere with data interpretation; an example is phenol red, which serves as a pH indicator in some media or fluorescence diagnostics such as organelle probes. Additionally, the cellular microenvironment not only affects fluorescence quantum yields but also the fluorophore’s emission wavelength maxima and bandwidth. Fluorophores have different photodynamic activities or photobleaching properties that may lead to shifts in emissions signals. There is a need for complete fluorescence spectra acquisition in cellular environments, which overcomes these limitations.

Fluorescence spectral bioimaging, using Fourier spectroscopy, allows spatial and spectral measurements that enable characterization of the fluorescence emission profile at every acquired pixel. A recent technique, it has been used successfully in fluorescence in situ hybridization applications to diagnose genetic defects by fluorescent staining, termed spectral karyotyping, of chromosomes (Schröck et al., 1996). Malik et al. (1996) have used spectral bioimaging microscopically to monitor protoporphyrin IX production after exogenous 5-aminolevulinic acid administration. Subsequent illumination of the cells caused photobleaching and photoproduct formation, with a distinctly different spectral peak appearing in the acquired spectrum.

In this study, a similar system was used to correctly identify the fluorophore motexafin gadolinium (MGd) in intracellular sites after MGd treatment. The biolocalization of MGd was confirmed by co-staining with organelle-specific probes and spectral overlay. MGd is a radiation enhancer that targets tumors and is now in human clinical trials (Rowinsky, 1999). To better understand the biochemical mechanisms that underlie these activities cell culture localization studies...
were performed. Here, wavelength-dependent microenvironmental changes, photobleaching, and subcellular fluorophore concentrations after Mg prevents treatment were determined in both the murine Rf-1 fibrosarcoma and EMT6 mammary sarcoma cell lines.

Materials and Methods

Chemicals. The synthesis and chemical characterization of Mg (also known as gadolinium texaphyrin, NSC #695238) used in this study has been described previously (Sessler et al., 1999). The compound was formulated in 5% aqueous mannitol, and the pH was adjusted to 5.5 with acetic acid to yield a final Mg concentration of 2.2 mM. Fluorescent probes for staining mitochondria (MitoTracker Green FM; MTG), lysosomes (LysoTracker red DND-99; LTR), Golgi apparatus (BODIPY FL C5-ceramide, C5-ceramide), endoplasmic reticulum (Rhodamine B, hexasyl ester, perchlorate; R6), and the nucleus (Hoechst 33342; HO342) were purchased from Molecular Probes (Eugene, OR).

Cells. Murine EMT6 mammary sarcoma and Rf-1 radiation-induced fibrosarcoma cell lines were maintained through established in vivo/vitro propagation procedures (Rockwell et al., 1972; Young et al., 1996). Cells were grown in Waymouth’s medium (MB752/1; Life Technologies, Grand Island, NY) supplemented with 14% fetal bovine serum (FBS; JRH Biosciences, Woodland, CA) and penicillin/streptomycin (Sigma, St. Louis, MO).

Colonies assay. EMT6 cells were cultured and harvested into 25-cm² plastic tissue culture flasks at a density of 200 cells/flask. Twenty-four hours was allowed for cell attachment to the flask at 37°C and 5% CO₂. MGd was added to yield a range of final concentrations from 0 to 150 μM. After 2 or 24 h MGd exposure the cells were washed three times with growth media without serum. The flasks were replenished with serum-supplemented media and returned to the incubator for an additional 6 to 10 days. The colonies were fixed with 10% buffered formalin and stained with crystal violet. Each determination was performed in triplicate and repeated twice for each data point.

Fluorescence Spectral Bioimaging. Female BALB/c mice, 7 to 8 weeks old, were purchased from Simonsen Laboratories, Gilroy, CA. Animals received care in compliance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health publication, 1996). The right flanks of the mice were shaved and depilated with Nair (Carter-Wallace, Inc., New York, NY) the day prior to tumor inoculation. EMT-6 cells (5–7 × 10⁵) were subcutaneously implanted into the right hind flanks of recipient mice. The tumor-bearing animals were studied 7 to 10 days after implantation. EMT-6 cells were washed three times with growth media without serum. The cells were seeded into 25-cm² plastic tissue culture flasks (Corning, NY) at a density of 2000 cells/flask. Twenty-four hours was allowed for cell attachment to the flask. MGd was added to yield a range of final concentrations from 0 to 150 μM. After 2 or 24 h MGd exposure the cells were washed three times with growth media without serum. The flasks were replenished with serum-supplemented media and returned to the incubator for an additional 6 to 10 days. The colonies were fixed with 10% buffered formalin and stained with crystal violet. Each determination was performed in triplicate and repeated twice for each data point.

Fluorescence Spectral Imaging. Female BALB/c mice, 7 to 8 weeks old, were purchased from Simonsen Laboratories, Gilroy, CA. Animals received care in compliance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health publication, 1996). The right flanks of the mice were shaved and depilated with Nair (Carter-Wallace, Inc., New York, NY) the day prior to tumor inoculation. EMT-6 cells (5–7 × 10⁵) were subcutaneously implanted into the right hind flanks of recipient mice. The tumor-bearing animals were studied 7 to 10 days after implantation. The chemical structure, absorption, and fluorescence profile of MGd is shown in Fig. 1. MGd has a large Soret band at 473 nm (log ε = 5.06). Due to its extended porphyrin aromatic conjugation system, the texaphyrin macrocycle has a low-energy band occurring at 740 nm (log ε = 4.59). The signature fluorescence emission band of MGd is centered at 758 nm and was used to monitor real-time MGd biolocalization in EMT6 mammary sarcoma-bearing mice. The animals were intravenously injected with MGd (40 μmol/kg) and then the tumor and surrounding

Excitation light was delivered from a xenon lamp (Cogen Light Technologies, Inc., Santa Clara, CA) via a multiband fiber terminating with a 470-nm interference filter (10-nm bandwidth; Oriel Corporation, Stratford, CT); a 695-nm long-pass filter (Oriel Corporation) was incorporated into the emission path. Each signal was averaged over 5 pixels.

Fluorescence Microscopy. The cells were seeded (4 × 10⁵) into Lab-Tek 10-cm² Flasket glass chamber slides (Nunc, Inc. Naperville, IL) containing 4 ml of media. The cells were allowed to incubate for 24 h to enable attachment to the flask. MGd was added to the media to yield a final concentration of 25 μg/ml and incubated for predetermined amounts of time. After the incubation, the cells were washed with serum-free media, overslipped, and sealed with nail polish. The specimens were viewed using a 63× oil-immersion objective via a Zeiss Axioplan-2 microscope. The SD200 SpectraCube system was coupled to the microscope via a C-mount. Both fluorescence and phase contrast micrographs were captured. All cell preparations were performed in triplicate.

Organellar-specific fluorescent probes were used: MTG, used to stain the mitochondria; LTR to stain the lysosomes; R6 to label the endoplasmic reticulum; C5-ceramide to stain the Golgi apparatus; and HO342 to stain the nucleus. Filter cubes optimized to match the spectral properties of the dyes and also integrated to match the coincident excitation wavelength of the Mg were chosen. A 360-nm bandpass filter (40 nm FWHM); a 400-nm dichroic filter and 420-nm long-pass filter were used to visualize HO342. A 485-nm bandpass (22-nm FWHM) filter, a 505-nm dichroic filter, and a 530-nm emission bandpass filter (30-nm FWHM) were used to confirm MTG’s fluorescence. LTR and R6 both required 535-nm (50-nm FWHM) excitation filters, 565-nm dichroic filters, and 590-nm long-pass emission filters. For visualizing the Golgi apparatus, a 470-nm bandpass filter (40-nm FWHM); a 505-nm dichroic filter, and a 515-nm long-pass filter were used. For MGd detection, the cells were excited using a 470-nm bandpass filter (40-nm FWHM), a 505-nm dichroic filter, and either a 515- or a 715-nm long-pass filter.

Caution was used when fluoroprobes were observed, since some organellar dyes become photosensitizing agents or photobleach (degrade) when subjected to high-intensity illumination, or low intensities for long exposure times (Twentyman et al., 1980; Woodburn et al., 1991). The samples were therefore illuminated with 63 mW/cm² from an HBO/100-W mercury lamp. Neutral red and lysotracker blue DND-22 were considered candidate lysosomotropic fluorescent probes for this study; however, they proved to be photolabile using the illumination conditions described here. Neutral red is particularly photoreactive; after localization to the lysosomes and following light exposure, the lysosomes ruptured and fluorescence dispersed. Lysotracker blue DND-22 readily photobleached in the presence of Mg, but did not stain well in the absence of serum.

The following dye concentrations and incubation times were used for staining specific organelles: 0.5 μg of HO342/ml for 10 min; 500 nM R6 for 30 min; 50 nM LTR for 1 h; 0.5 mg/ml BODIPY FL C5-ceramide for 10 min; and 250 nM MTG for 30 min. Phase contrast and fluorescence images were stored electronically and color processed using the EasyFISH software (Applied Spectral Imaging).

Results

In Vivo Fluorescence Imaging. The chemical structure, absorption, and fluorescence profile of Mg is shown in Fig. 1. Mg has a large Soret band at 473 nm (log ε = 5.06). Due to its extended porphyrin aromatic conjugation system, the texaphyrin macrocycle has a low-energy band occurring at 740 nm (log ε = 4.59). The signature fluorescence emission band of Mg is centered at 758 nm and was used to monitor real-time Mg biolocalization in EMT6 mammary sarcoma-bearing mice. The animals were intravenously injected with Mg (40 μmol/kg) and then the tumor and surrounding...
normal skin were illuminated with 470-nm light, corresponding to the Soret band, 5 h postinjection. The resultant fluorescence emission signals were captured with a CCD camera coupled to an interferometer, and then the signal was Fourier-transformed, allowing spectral identification at each pixel (Fig. 2). Integration of the signal over the emission captured range (720–780 nm), for four animals, and subtraction of the endogenous fluorescence revealed a 6-fold enhancement in the tumor relative to surrounding normal tissue.

**MGd Photobleaching.** The fluorescence quantum yield of MGd is low. However, it can be used for detection and quantitation due to the large extinction coefficient at the excitation wavelength of 473 nm (log ε = 5.06), a large Stokes shift (758 nm), and a detection system that exhibits optimal quantum efficiencies in the desired wavelengths. Because irreversible destruction or photobleaching of the fluorophore limits the illumination intensity and time of exposure, studies were undertaken to determine the effect of illumination on MGd photobleaching. An illumination intensity of 63 mW/cm² caused no significant photobleaching, whereas using an intensity of 209 mW/cm² did (Fig. 3). Interestingly, repeating an illumination at 63 mW/cm² did not significantly change the fluorescence profile and emission (data not shown).

**Uptake Kinetics.** The localization of MGd in EMT6 and Rif-1 cells was evaluated for incubation periods up to 72 h in medium supplemented with 14% FBS. No inherent cytotoxicity, measured using a clonogenic assay, was observed using MGd concentrations up to 150 μM in serum supplemented medium (data not shown). Using a 72-h incubation time in serum-free medium the IC₅₀ was 25 μM so for serum-free media experiments incubation periods were confined to 24 h or less.

A time-dependent increase in intracellular MGd fluorescence was seen in both cell lines. Distinct, punctate localization patterns were observed that became more defined with incubation time. A typical fluorescence pattern for EMT6 cells is shown in Fig. 4b that was obtained after incubation of MGd for 4 h. MGd, noted by its characteristic 758-nm profile, was not present in the nuclei. Uptake, based on the use of the previously mentioned organelle probe stains, appeared to occur in the lysosomes, endoplasmic reticulum, and some mitochondria. A large fluorescence signal was detected in the perinuclear region, suggestive of localization of MGd within the Golgi apparatus. Generally, MGd was not present in the nuclei; however, 15% of cells showed nuclear association in the EMT6 cell line after long incubation intervals. In this small subset, as much as 40% of MGd was present within the nucleus.
nuclei (Fig. 5, b and c). The nuclear membranes were well defined, and fluorescent areas were seen in a compartmentalized manner throughout the cytoplasm.

The temporal uptake of both cell lines, with and without serum supplementation, is shown in Fig. 6. It is important to note that the obtained spectra were taken from typical punctate localization sites within the cells and, as such, were not reflective of the total cellular content. The fluorescence intensity significantly increased with time; little signal was detected after 2 h, and was sparsely located throughout the cytoplasm. Large signals were clearly defined at the later time points. Omitting serum from the medium increased MGd uptake.

Fig. 3. Fluorescence spectra of MGd-treated Rif-1 cells. Cells were incubated with 25 μg/ml MGd and then imaged at 24 h with an illumination intensity of 63 mW/cm² (●) followed by another acquisition using 209 mW/cm² (○). The data reflect fluorescence pixels (3 × 3) within a punctate fluorescent area compared with background (background, open symbols).

Staining with Organelle Probes. The subcellular localization of each of the studied organelle probes was confirmed based on the unique staining patterns of the Rif-1 and EMT6 murine cell lines. Typical localization patterns for the murine radiation-induced fibrosarcoma cell line (Rif-1) are illustrated in Fig. 7. In Fig. 7b, the cells were sequentially stained with LTR, C5-ceramide, and HO342. The red fluorescent LTR stains the lysosomes, the green C5-ceramide targets the Golgi apparatus, and the blue fluorescent HO342 accumulates in the nuclei. Three exposures, with specific filter cubes matched to each probe, were acquired and then overlaid to yield the depicted image. Figure 7c denotes the distinct green curlicue staining characteristic of the mitochondria, and Fig. 7d shows the red fluorescent R6 accumulating in the endoplasmic reticulum.

The intracellular fluorescence emission profiles of each of the organelle probes was acquired using spectral bioimaging. The normalized fluorescence emission spectra obtained using filter cubes matched to each of the probe’s specific spectral properties are shown in Fig. 8. The emission maxima for C5-ceramide, HO342, MTG, R6, and LTR were 522, 527, 533, 599, and 612 nm, respectively. C5-ceramide possesses concentration- and environment-dependent properties. At high concentrations, C5-ceramide forms longer wavelength excimers (excited state dimers) in the Golgi apparatus that result in a wavelength shift from 522 to 637 nm (Fig. 8b). R6 fluorescence can also be observed, but with less fluorescence output, with the MGd/C5-ceramide filter cube, and yields an emission maximum of 584 nm.

Colabeling with Organelle Probes. After exposure of the cells to MGd for varying times, organelle-specific fluoroprobes were added for colocalization studies. It was necessary to wash the cells with serum-free medium before incubation with the organelle-specific probes, because most probes, and also the MGd, are planar aromatic systems and may interact noncovalently and cause unwanted artifacts that change biolocalization. Dichroic and emission filters were used because, for multispectral labeling, it is ideal that the dye undergoing analysis and the organelle-specific flu-

Fig. 4. MGd localization within EMT6 cells. EMT6 cells were treated for 4 h with 25 μg/ml MGd. The transmission and fluorescence micrographs are illustrated in a and b, respectively. The fluorescence emission spectra, analyzed at various regions, are shown in c.
orophore have the same coincident wavelength custom excitation. Special attention was given to the long-pass filters, so the different fluorophores could be simultaneously spectrally resolved at every pixel within the CCD array study area.

Colocalization labeling using the various fluorophores confirmed MGd uptake was within the lysosomes and endoplasmic reticulum, with less partitioning into the mitochondria and Golgi apparatus at early incubation times (<24 h) in both the EMT6 and Rif-1 cell lines. At longer incubation times there was more MGd present within the Golgi apparatus. For example, Fig. 9b shows the overlay staining of MGd, HO342, and LTR in Rif-1 cells. The cells were incubated with 25 μg/ml MGd for 6 h. The overlay involved the three filter cube sets responsible for HO342, LTR, and 715-nm long-pass MGd. The nuclei were distinctly defined by the blue staining of HO342. Both LTR (red) and MGd (yellow) staining were in punctate areas, often overlapping; several areas were distinctly isolated from each other. Spectral analysis for four different extranuclear pixel areas using the 520 nm long-pass filter set, is shown in Fig. 9d. The blue line denotes a distinct yellow pixel area from Fig. 9b, revealing the presence of MGd. The other lines represent colocalization areas of MGd and LTR. It is apparent that MGd was present in nearly all lysosomes, but not all of the MGd was associated with lysosomal uptake.

Longer incubation times (72 h) indicated higher partitioning within the Golgi apparatus (data not shown). Spectral analysis using the 520-nm long-pass filter set, used to captured both the C₅-ceramide and MGd fluorescence, revealed areas of colocalization of the C₅-ceramide with MGd. It is interesting to note that not all the Golgi showed MGd association and that which did, did not exhibit excimer formation (data not shown).

**Discussion**

Motexafin gadolinium, or MGd, is a paramagnetic, aqueous-soluble expanded pentadentate porphyrinoid that is selective for neoplastic cells (Young et al., 1996). MGd alone, using the conditions described here, has no observed therapeutic impact. However, radiation enhancement with MGd in combination with radiation therapy has been seen in single and multiradation studies in experimental tumor models (Miller et al., 1999; Minamikawa et al., 1999). MGd is presently in a phase III clinical trial for patients undergoing whole brain radiation therapy for brain metastases (Rowin-
and is also being evaluated in nonclinical models as a chemosensitizer with doxorubicin and bleomycin.

Intracellular localization studies may provide mechanistic insights into MGd’s mode of action as both a radiation enhancer and a chemosensitizer. Some discordance has been observed when assessing in vitro radiation responses as MGd displayed minimal radiation enhancement in in vitro clonogenic assays, whereas good tumor responses were recorded in murine models (Miller et al., 1999). It is hoped that interferometric Fourier fluorescence microscopy may aid in defining the importance of tumor-host interactions versus intracellular interactions.

Noninvasive spectral bioimaging of EMT6 tumor-bearing mice showed enhanced localization of MGd in the sarcoma compared with the surrounding normal tissue, resulting in an enhancement ratio of 6 to 1. The latter fluorescent result was in accord with previous studies using magnetic resonance imaging to detect the paramagnetic gadolinium coordinated into the central core of the macrocycle and 14C-labeled MGd (Miller et al., 1999; Minamikawa et al., 1999). The latter radiolabeled biodistribution study performed in SMT-F mammary tumor-bearing mice yielded a tumor-to-muscle ratio of 8.5 to 1 at 5 h after intravenous administration of 9.9 μmol MGd/kg (Miller et al., 1999). It may also be possible to study the intracellular localization of MGd using subcellular fractionation with radiolabeled MGd. However, cultured cells are difficult to fractionate, they are more problematic than tissues due to differences in cytoskeletal organization, and additionally, with subfractionation it is difficult to cleanly resolve many organelles into distinct fractions (Howell et al., 1989; Pasquali et al., 1999), so alternative more facile approaches to discerning intracellular localization sites of drugs are sought.

The uptake of MGd within EMT6 and Rif-1 cells, assessed using interferometric Fourier fluorescence microscopy was immediate. Retention increased significantly with time and was more pronounced in serum-free media. MGd partitioned within the lysosomes, endoplasmic reticulum, and, to a lesser extent, the Golgi apparatus and mitochondria in serum-sup-

**Fig. 7.** Representative staining of Rif-1 cells with fluorescent organelle-specific probes. In the transmission micrograph in a, cells were stained with fluorescence probes (b) specific for the nuclei (blue), Golgi apparatus (green), and lysosomes (red). The fluorescence micrograph in c depicts the mitochondria as green and the nuclei as blue. The endoplasmic reticulum is illustrated by the red staining in d, with the nuclei appearing blue.

**Fig. 8.** In vitro normalized fluorescence emission spectra of organelle-specific fluoroprobos. The emission maxima for H0342, C5-ceramide, and LTR are shown in a. C5-ceramide, at high concentrations in the Golgi apparatus, forms longer wavelength excimers that result in a wavelength shift from 522 nm, C5-ceramide (a), to 637 nm, C5-ceramide (b). The normalized fluorescence emission spectra of MTG and R6 are shown in b. R6 fluorescence was observed with two filter cubes, the custom filter cube, R6 (a), and the MGd/C5-ceramide filter cube, R6 (b).
implemented medium. Only in 15% of EMT6 cells, at 48 h, was MGd observed in the nucleus. In serum-free medium, more MGd was partitioned within the mitochondria.

Interferometric Fourier fluorescence microscopy is a potentially valuable technique that will resolve subcellular localization of fluorophores using known organelle-specific markers. Fluorescence is often wavelength-dependent, and varies with alterations in kinetic and physiological factors with different cellular compartments. The latter cannot be analyzed from single-point determinations as is obtained using fluorescence filter technology. Monitoring the fluorescence emission range not only aids in subcellular localization studies but is also valuable in assessing cellular changes using different biochemical markers. Experiments of this kind are currently in progress in this laboratory.

References


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Fig. 9. Colocalization of MGd within lysosomes. Rif-1 cells were incubated with MGd for 6 h in serum-supplemented medium and then incubated with LTR and HO342. A transmission micrograph is shown in a, and a multiple exposure image obtained with filter sets appropriate for HO342 (blue), LTR (red), and MGd (yellow) is shown in b. Spectral analysis of four extranuclear pixel areas is depicted in c.