Subcellular Distribution of the Anticancer Drug Mitoxantrone in Human and Drug-resistant Murine Cells Analyzed by Flow Cytometry and Confocal Microscopy and Its Relationship to the Induction of DNA Damage

Paul J. Smith, Hazel R. Sykes, Mary E. Fox, and Isla J. Furlong

MRC Clinical Oncology and Radiotherapeutics Unit, MRC Centre, Hills Road, Cambridge, CB2 2QH, United Kingdom

ABSTRACT

Flow cytometry and laser scanning confocal imaging have been used to analyze the uptake of the anticancer topoisomerase II poison mitoxantrone by intact mammalian cells and the results correlated with the induction of DNA damage. Unlike Adriamycin, mitoxantrone displays only minimal levels of red fluorescence when excited at 514 nm wavelength. However, using these excitation and emission conditions, flow cytometry could detect low levels of fluorescence in human transformed fibroblasts exposed to high concentrations (5–20 μM) of mitoxantrone for 1 h. Over this dose range whole cell fluorescence was a function of cell size and increased with drug concentration while drug-induced DNA-protein cross-linking showed saturation. Confocal microscopy revealed the time- and dose-dependent appearance of fluorescence, interpreted here as reflecting the disposition of drug molecules, preferentially within the cytoplasm, nuclear membrane, and nucleoli. This pattern contrasted with the intense intranuclear fluorescence observed in Adriamycin-treated human cells. Loss of the nuclear membrane during mitosis resulted in an apparent increase in chromatin-associated fluorescence. Photon counting procedures revealed a predominantly cytoplasmic, possibly lysosomal, location for fluorescence from human cells exposed for 1 h to a low but cytotoxic concentration (0.1 μM, yielding approximately 90% cell kill) of mitoxantrone. At this low concentration, human cells displayed minimal levels of DNA strand cleavage or DNA-protein cross-linking. Murine cells, displaying mitoxantrone resistance as part of the P-glycoprotein-mediated multidrug resistance phenotype, showed specific extinction of mitoxantrone-associated fluorescence from the nucleus but not from within extranuclear compartments. The study demonstrates the feasibility of high resolution studies on the intracellular distribution of mitoxantrone in intact living cells. We suggest a mechanism by which cytoplasmic sequestration of mitoxantrone may be important in determining the responses of normal and multidrug-resistant cells as they attempt to progress through mitosis.

INTRODUCTION

Mitoxantrone is an anticancer anthraquinone drug, structurally related to the DNA intercalating anthracycline antibiotics. It is used clinically in the treatment of non-Hodgkin’s lymphomas, acute myeloid leukemias, and advanced breast cancer (1–3). Compared with other anticancer intercalating agents mitoxantrone is unusually potent (4, 5). It is probable that various properties of the drug contribute to its high cytotoxicity, including the unusual mode of binding to DNA, the effects on DNA conformation, the induction of persistent DNA damage, the long-term inhibition of DNA synthesis, and the persistence of the drug within cells. The principal aim of the present study was to identify important biological targets for the cytotoxic action of mitoxantrone. The approach used was to apply high resolution imaging methods to follow the uptake and subcellular distribution of drug in mammalian cell lines and to relate the findings to the cytotoxic and DNA-damaging effects of the agent.

While mitoxantrone has been shown to bind to DNA through intercalation (6, 7), the alkylamino side chains are known to bind electrostatically to the anionic exterior of the helix and prevent full intercalation (8–10). Some investigators have reported G-C base pair specificity (9, 11), while others report no high degree of sequence specificity (8, 12). The mode of DNA binding may explain the ability of mitoxantrone to cause condensation and compaction of chromatin (13) together with the disturbance of DNA-protein interactions (14). Importantly, mitoxantrone traps the cleavable complex that forms between DNA and the nucleus-located enzyme DNA topoisomerase II (15–17), although it has been proposed that other types of DNA lesions may arise from damage by free radicals (18). Studies using radiolabeled drug have revealed the extensive binding of mitoxantrone to nuclear and cytoplasmic structures and the persistence of drug molecules within cells (19, 20). It is thought that DNA damage arising from persistent topoisomerase II trapping by mitoxantrone may explain the observed long-term inhibition of DNA synthesis, arrest of cells in the G2 phase of the cell cycle, and cell death (5, 21).

The natural fluorescence of agents such as the anthracyclines has enabled uptake studies to be performed using flow cytometry or fluorescence microscopy (22–25). These two approaches are applicable to intact, viable cells and enable distribution of drug molecules to be monitored both throughout cell populations and within intracellular compartments. Although mitoxantrone shows minimal fluorescence upon excitation, an indirect fluorochrome competition method (26) has been used to detect the binding of mitoxantrone to nuclear DNA in single cells (16). In an attempt to devise a direct imaging method for mitoxantrone this preliminary study has addressed the feasibility of using flow cytometry and confocal scanning laser microscopy to study mitoxantrone uptake and distribution in living cells.

The essential feature of confocal imaging of fluorescent specimens is that light from out-of-focus regions is eliminated from the detection system (27). Thus only regions of the specimen within a narrow depth of focus are imaged. This approach has recently been used for high resolution studies on the subcellular distribution of daunorubicin (25). An image of fluorescence can be built up, with appropriate filtration for background noise, from within a defined volume of the specimen as determined by the size of the confocal aperture within the optical array. The use of photon counting methods enables low levels of fluorescence to be detected. Electronic processing of the stored image provides an accurate measure of fluorescence intensity and its 2-dimensional distribution within the optically sectioned volume. Indeed, by altering the plane of focus, a series of images can be accumulated and their projections used to recreate a...
3-dimensional image representing the distribution of fluorescence intensity throughout a specimen.

The feasibility of direct imaging of mitoxantrone within intact cells has been studied here using human transformed fibroblasts (28) and murine cell lines which differ in their intrinsic sensitivities to mitoxantrone. The murine cell lines include two blasts (28) and murine cell lines which differ in their intrinsic damaging potential.

Rapid efflux on drug accumulation, distribution, and DNA-protein complexes were precipitated by the addition of KCl. Precipitates were subjected to a repeated washing process of solubilization and reprecipitation. DNA-protein cross-linking is expressed as precipitable radioactivity as a percentage of the total radioactivity in the sample.

Flow Cytometry. Following drug treatment of monolayer cultures, cells were detached and resuspended in fresh medium and analyzed immediately using a flow cytometer incorporating an Innova 70 argon laser (Coherent) tuned to 514 nm. Signals for forward and 90-degree light scatter and fluorescence above 550 nm were collected for 1×10^4 cells using the 90-degree light scatter parameter as the master signal. Fluorescence intensities for populations of single cells were derived by computer analysis.

Confocal Laser Scanning Microscopy. The confocal imaging system used was a Bio-Rad MRC-600 (Bio-Rad Microscience Ltd., Hemel Hempstead, United Kingdom) scanning assembly incorporating a 25 mW argon ion laser and coupled to a Nikon Optiphot II fluorescence microscope. Excitation was at 514 nm wavelength. Cells were grown at a density of 5×10^4 cells/well as a monolayer on autoclaved glass coverslips in 6-well plates. Cells were treated with mitoxantrone for various incubation times. Inverted coverslips were mounted on glass slides in PBS, the coverslips being supported on lacquer tiers to prevent the cells from being compressed. The slides were examined immediately under the confocal laser scanning microscope with no neutral density filtration, confocal apertures of 2 to 7 and a 488 nm immersion objective lens. Successive images of the cells were stored after Kalman filtration on optical cartridge and photographs taken subsequently using Ifford FP4 film. In all cases, the operating conditions were such that detectable images could not be obtained for cell samples not treated with drugs.

RESULTS

Induction of DNA Damage in Transformed Human Fibroblasts. Drug-induced DNA strand breakage (including breakage arising from trapped cleavable complexes) could be detected in cells treated with low concentrations (0.16–2.5 μM) of mitoxantrone with evidence of saturation of lesion frequencies at drug concentrations between 1.25 and 2.5 μM (Fig. 1). X-irradiation calibrations (see “Materials and Methods”) indicated approximately 10 Gy equivalents of damage at 2.5 μM mitoxantrone, although these levels of strand breakage observed were at the upper limits of the denaturation assay used. Fig. 1 indicates that similar increments in the levels of DNA-protein cross-linking were induced at low mitoxantrone doses with evidence of saturation within a similar 2–4 μM dose range.

Assessment of mitoxantrone sensitivity in parallel clonogenic assays using the method described previously (5) indicated a D_0 value of 27.3 nm mitoxantrone (1 h drug exposure) by computer fitting (35) the combined clonogenic survival data from three experiments (data not shown). The 10% survival dose for MRC5CVI cells was within the range 80–100 nm mitoxantrone. Thus the detection of significant initial levels of DNA...
Mitoxantrone-induced Cellular Fluorescence Monitored by Flow Cytometry. Early in this study we observed that mitoxantrone-treated cells showed low levels of fluorescence. Fluorescence could not be observed directly by normal epifluorescence microscopy but was detectable using the laser scanning confocal microscope operated under high intensity for the incident beam and high photomultiplier gain settings. Using an excitation wavelength of 514 nm and monitoring green fluorescence. The fluorescence is interpreted here as reflecting the subcellular distribution of drug molecules. Initially we sought to confirm these observations by conventional flow cytometry using an excitation and emission configuration similar to that used on the confocal microscope. A dose-dependent increase in the mean, median, and modal channels of fluorescence intensity for asynchronous MRC5CVI cell populations was evident (Table 1). Even at a high mitoxantrone concentration of 20 μM, giving a 2.7-fold increase in mean fluorescence intensity versus that of control cells, there was an overlap of the fluorescence distributions for treated and control cells (Fig. 2). Fig. 2 also shows the distribution of fluorescence intensity throughout an asynchronous population as a function of cell size monitored by right-angle light scatter. Fluorescence intensity clearly increases with cell size.

Confocal Fluorescence Microscopy. The fluorescence observed in drug-treated human cells was clearly detectable following exposure to 1 μM mitoxantrone (Fig. 3b) and increased ~3-fold when the external drug concentration was raised to 5 μM (Fig. 3c). Weak fluorescence could be detected after exposure to a low mitoxantrone drug concentration of 100 nm by the use of photon counting and image accumulation (Fig. 3a). Fluorescence was primarily observed within cytoplasmic inclusions with a dose-dependent appearance of nuclear membrane, nucleolar, and general cytoplasmic staining. This pattern was in distinct contrast to the pattern observed in Adriamycin-treated cells (Fig. 3d) which displayed intense staining of the nuclear membrane and perinucleolar regions of the nucleus. Image analysis of cells exposed to 1 μM drug concentrations revealed >4-fold lower levels of nuclear fluorescence in mitoxantrone-treated cells compared with Adriamycin-treated cells.

By varying the time of incubation with a standard 20 μM drug dose the pattern of mitoxantrone uptake was determined. Fig. 4 shows images of MRC5CVI cells after 15-min, 1-, and 2-h incubation periods. Fluorescence intensity increased with incubation period, stabilizing at around 1 h. After 15 min, fluorescence was apparent within cytoplasmic inclusions which tended to group in a region adjacent to the nucleus. Nuclear membranes and nucleolar-like bodies were clearly defined and, as the period of incubation increased, there was a preferential increase in the fluorescence intensity of nuclear-associated structures. A Z-axis series for the fluorescence distribution of mitoxantrone in MRC5CVI cells was generated (Fig. 5) by sectioning at approximately 2 μm intervals. Z-sections adjacent to the surface of attachment (Fig. 5a) revealed fluorescence associated with the spreading edges of cytoplasm. The successive images confirmed the intracellular distribution noted above. The sections shown in Fig. 5 include a mitotic cell, within which the highly fluorescent cytoplasmic inclusions are less apparent and the condensed chromatids are homogeneously stained.

Mitoxantrone-associated Intracellular Fluorescence in Multidrug-resistant Cells. To determine the extent to which the cytoplasmic and nuclear compartments are protected by the effects of active drug efflux we have compared the intracellular fluorescence patterns in four murine cell lines, two of which display multidrug resistance and reduced cellular accumulation of a range of xenobiotic molecules (31, 32, 37). Initially we confirmed that the EMT6/AR1.0 (D50 18 nm) and...
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EMT6/CR1.0 (D50 53 nM) cell lines demonstrated (Fig. 6) enhanced resistance to continuous exposure to mitoxantrone when compared with the EMT6/P (D50 2.7 nM) and EMT6/MR1.0 (D50 2.7 nM) cell lines. The most resistant murine line, EMT6/CR1.0, also demonstrates reduced DNA-protein cross-linking (approximately 60%) compared with the parental line when exposed to high mitoxantrone doses for 1 h (Fig. 7). Fig. 8 shows that high-dose (20 μM) mitoxantrone-treated parental EMT6/P and methotrexate-resistant EMT6/MR1.0 cells demonstrated patterns of fluorescence similar to those of MRC5CVI cells. The two multidrug-resistant cell lines showed complete extinction of nuclear fluorescence, no nuclear membrane staining, and a reduced background of cytoplasmic fluorescence, but they also showed the maintenance of the highly fluorescent cytoplasmic inclusions.

DISCUSSION

Previous studies on the intracellular pharmacology of mitoxantrone have been hampered by the lack of noninvasive methods for determining the distribution of drug molecules within intact, living single cells and within viable cell populations. Despite the low level of intrinsic fluorescence shown by the anthraquinone, we have demonstrated here that drug-treated cells display low levels of fluorescence detectable by the complementary techniques of flow cytometry and confocal laser scanning microscopy. We suggest that intracellular fluorescence reflects, at least in part, the distribution of drug molecules. The application of confocal microscopy has enabled the cellular compartments for mitoxantrone accumulation to be identified. At low, biologically relevant concentrations of mitoxantrone we have observed a predominantly cytoplasmic deposition of drug. Intracellular location of drug was detected at higher concentrations which induce saturating levels of DNA-protein cross-linking. The multidrug resistance phenotype appears to result in an active protection of the nuclear compartment despite the accumulation of drug within cytoplasmic compartments evidently not accessible to drug efflux pathways.

Preliminary attempts, not described here, to demonstrate mitoxantrone fluorescence in neutral aqueous solutions with excitation at 514 nm revealed only very low levels of fluorescence. Despite the essentially nonfluorescent nature of mitoxantrone, preliminary studies also showed that aqueous suspensions of mitoxantrone adsorbed onto a silica matrix showed an emission...
Fig. 4. Time-dependent appearance of intracellular fluorescence, detected by laser scanning confocal microscopy, within MRC5CVI fibroblasts exposed to 20 μM mitoxantrone. a, b, and c, 20-min, 1- and 2-h exposure periods, respectively. Bars, 25 μm.

signal when scanned using a confocal imaging system operated under high intensity for the incident beam and high photomultiplier gain settings. The fluorescence observed in mitoxantrone-treated cells was at the limits of detection for biologically relevant drug concentrations. However, the application of a photon counting procedure allowed the collection of fluorescence images from cells treated with 0.1 μM mitoxantrone. The flow cytometric study has shown that whole cell fluorescence intensity is a function of cell size and shows a linear relationship with mitoxantrone concentration. The microscopy study reveals that the fluorescence intensities of the cytoplasmic and nuclear compartments also continue to increase as a function of mitoxantrone concentration over dose ranges which yield saturating levels of DNA-protein cross-linking and strand cleavage detected under alkaline conditions.

The fluorescence associated with mitoxantrone in these experiments is unexpected since this agent is not known to demonstrate measurable fluorescence in solution. The nature of the fluorescent species detected in mitoxantrone-treated cells is not clear but may be a product of singlet-singlet transitions by free mitoxantrone or mitoxantrone-macromolecular charge transfer complexes detected only under the intense laser light. Under these conditions the quantum yield of fluorescence from either free mitoxantrone or a charge transfer complex may be increased to detectable levels. Such fluorescence would be dependent on the ionic nature and pH of the environment. Alternatively the laser light may directly induce the formation of a mitoxantrone/macromolecular or macromolecular covalent adduct with fluorescence properties detectable under the conditions used. Thus it is possible that the intensity of fluorescence may reflect the distribution and disposition of specific structures, ligands, and microenvironments (38, 39). For example, the nonfluorescent acridine 4’-(9-acridinylamino)methanesulfon-m-anisidide appears to be capable of covalently binding to the sulfhydryl groups in the RBC membrane, with the production of fluorescent 4’-(9-acridinylamino)methanesulfon-m-anisidide-protein adducts (40). Using 14C labeling and trichloroacetic acid precipitation methods it has previously been shown that mitoxantrone also binds to macromolecules in cells (19) and can persist within cytoplasmic compartments of cultured cells following the removal of drug from the external medium (20). Clearly, fractionation experiments will be required to characterize the fluorescence properties of mitoxantrone within different cellular compartments. Importantly, the present study reveals that the intracellular distribution of fluorescence for mitoxantrone and Adriamycin are different, underlining the advantage of direct imaging methods in drug uptake studies.

The optical sections of mitotic cells treated with mitoxantrone suggest that the drug has affinity for DNA and fluoresces even when bound. Indeed, we have also observed that isolated methanol/acetic acid-fixed human chromosomes fluoresce, under the same excitation/emission conditions used in this study, when mounted in aqueous solutions of mitoxantrone (data not shown). Three-dimensional image reconstruction methods could be used to determine whether the fluorescence intensity of mitotic figures reflects the condensation of less intensely staining structures into smaller scanned volumes or an absolute increase in the intensity of chromatin fluorescence. Optical sections of interphase nuclei reveal a preferential location of fluorescence within nucleolar-like structures perhaps...
reflecting the proposed base pair preference of the intercalator (9, 11) and the GC-rich nature of nucleolar DNA. These structures become fluorescent within minutes of drug exposure in human cells and have been observed in various cell types including murine cells, human adenocarcinoma, and small cell lung cancer cells.

The rapid drug efflux mechanism associated with classical multidrug resistance serves to prevent the appearance of fluorescence within murine cell nuclei but not within cytoplasmic inclusions. Despite the lack of nuclear fluorescence in EMT6/CRI.0 cells treated with a high dose of mitoxantrone, substantial levels of DNA-protein cross-linking could be detected. This suggests that relatively low intranuclear mitoxantrone concentrations are active in the induction of topoisomerase II-related DNA damage. Comparing the dose dependencies for DNA damage induction and the appearance of nuclear fluorescence in human cells again suggests that lesion saturation occurs at relatively low drug concentrations (approximately 1–2.5 μM range) which are not saturating for intranuclear fluorescence. The patterns for lesion saturation are consistent with a critical dependence on an auxiliary factor, namely topoisomerase II (17), for lesion induction. Thus, we suggest that the toxicity of mitoxantrone is primarily determined by the topoisomerase profile of a cell and the protection of that target, for example, by rapid drug efflux mechanisms.

The nature of the structures associated with the cytoplasmic inclusions is unknown, although the structures appear to correspond in size and location with lysosomes (41). Indeed, accumulation of agents such as Nile blue (41), acridine orange

Fig. 5. Series of sequential optical sections, at approximately 2-μm intervals apart, showing the distribution of intracellular fluorescence through a group of MRC5CVI cells treated with 20 μM mitoxantrone for 1 h. Note the mitotic cell in the upper right quadrants. Bars, 25 μm.

Fig. 6. Sensitivity of murine cell lines to mitoxantrone. Data are arithmetic means of triplicate determinations ±SE (bars) from a single representative experiment. ○, EMT6/P parental line; △, EMT6/MR1.0; ▲, EMT6/AR1.0; ●, EMT6/CR2.0.

Fig. 7. Mitoxantrone-induced DNA-protein cross-linking in murine cells. Data are arithmetic means of 4 determinations ±SE (bars). ○, EMT6/P; ●, EMT6/CR2.0.
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Fig. 8. Intracellular fluorescence, detected by laser scanning confocal microscopy, within normal and drug-resistant murine cells following a 1-h exposure to 20 \( \mu \text{M} \) mitoxantrone. a, EMT6/P parental line; b, EMT6/MR1.0; c, EMT6/AR1.0; d, EMT6/CR2.0. Bars, 25 \( \mu \text{m} \).

(39), and Adriamycin (42) within lysosomes have been noted previously. A high level of sequestration of mitoxantrone within such inclusions may result from partition in membrane lipids (39, 41). Deposits of drug which are not readily exchangeable with other cell compartments would be expected to persist and not be subject to rapid efflux from cells via a P-glycoprotein-dependent pathway (29). Previous studies have also indicated the long-term retention of mitoxantrone in human carcinoma cells (19, 20). We have observed that the fluorescent cytoplasmic inclusions are preferentially retained upon posttreatment incubation and specifically reduced in frequency in mitoxantrone-resistant adenocarcinoma cells.* Thus, the degree of cytoplasmic retention may be a secondary factor in determining cellular sensitivity to mitoxantrone and possibly represents a slow releasing pool of drug which may contribute to the chronic topoisomerase II trapping observed with this drug (5).

Dietel et al. (43) have reported an intensive formation of surface vesicles associated with the emergence of resistance to mitoxantrone in cultured gastric carcinoma cells. These vesicles consisted of an inner and an outer double membrane and were almost absent in sensitive cells. Following mitoxantrone treatment the blue drug could be observed in the vesicles using interference contrast microscopy of formalin fixed cells (43) suggesting that resistance may arise by a process involving intracellular compartmentalization. Accordingly, it was proposed that when mitoxantrone approaches a resistant cell it is linked to a binding or receptor protein, not present in sensitive cells, and rapidly released by exocytosis (43). It is likely that the fluorescent cytoplasmic inclusions observed in the present study are distinct from the vesicles observed in resistant cells. Indeed, optical sectioning showed that the cytoplasmic inclusions were located within the body of the cytoplasm adjacent to the nucleus and were present in similar numbers both in MRC5CVI cells and in mitoxantrone-sensitive transformed fibroblasts. The cytoplasmic inclusions also persisted within cells for at least 24 h following removal of drug from the external medium (data not shown).

The present study shows that following exposure to low levels of mitoxantrone, fluorescence is predominantly cytoplasmic and multidrug resistance gives rise to low intranuclear levels of fluorescence. Thus it is possible that biologically relevant levels of intranuclear drug, inducing low initial levels of persistent DNA damage which can effect cell kill (5), may not be imaged by this confocal method. However, the comparison of multidrug-resistant cells and “normal” cells in terms of initial levels of damage and cellular sensitivity is complex. Our findings are consistent with a previous report that normal and multidrug-resistant human cell lines, with large differences in toxicity

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* M. E. Fox and P. J. Smith, unpublished data.

5 P. J. Smith, unpublished data.
responses to mitoxantrone, may differ by a matter of only two-fold in the initial levels of DNA-protein cross-linking induced (16). It cannot be assumed that a simple relationship exists among the level of nuclear bound drug, DNA-protein cross-linking, and intranuclear fluorescence intensity. For example, the level of drug in the nuclei of multidrug-resistant cells may be below a threshold for efficient charge transfer complex formation but at a sufficient level to induce DNA-protein cross-linking. Alternatively, at high drug concentrations damage induction may become saturated or inhibited (see "Results" and Ref. 16) despite progressive binding of the drug to DNA.

In interpreting the cytoplasmic fluorescence seen at low drug concentrations as being sequestered drug, it is reasonable to consider whether this drug pool can be mobilized. We have observed that mitotic cells (Fig. 5) show a reduced level of cytoplasmic fluorescence compared with the bright fluorescence show by chromosomal material. Thus it is possible that the sequestered cytoplasmic pool is released upon entry into mitosis resulting in efficient cell kill due to unimpeded access to chromatin and peak levels of the target enzyme DNA topoisomerase II (44). In the case of multidrug-resistant cells, any sequestered drug released would be rapidly removed from the cell resulting in progression through mitosis and a reduced drug burden for daughter cells. Studies with synchronized cells may help to determine such a process could contribute to the large difference in mitoxantrone toxicity between normal and multidrug-resistant cells despite similar levels of cytoplasmic loading.

The present study indicates the feasibility of using laser scanning confocal microscopy to analyze biologically relevant levels of anthraquinones in human cells. This noninvasive approach retains important features of cellular architecture which may distinguish the anthraquinones from the anthracyclines in terms of subcellular distribution. Such a single cell analytical method is ideally suited to the analysis of heterogeneity within tumor biopsy specimens and could be used to monitor the functional expression of multidrug resistance with respect to the ability to condense nucleic acids. Proc. Natl. Acad. Sci. USA, 83: 6302-6306, 1986.


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