Confocal microscopy of idarubicin localisation in sensitive and multidrug-resistant bladder cancer cell lines

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Summary
Idarubicin is a highly lipophilic anthracycline and appears effective against tumours resistant to conventional anthracyclines. Confocal microscopy demonstrates predominantly cytoplasmic idarubicin accumulation. This distribution is unaltered by resistance status or the resistance reversing agent verapamil. Our results contrast with studies on conventional anthracyclines and suggest that nuclear accumulation may not be a prerequisite for anthracycline cytotoxicity.

Keywords: idarubicin; epirubicin; bladder cancer; confocal microscopy; verapamil; multidrug resistance

Anthracyclines are widely used chemotherapy agents. Thought to act primarily as DNA intercalators (Plosker and Faulds, 1993) they are effective against a wide range of tumours. However, their clinical utility is limited by multidrug resistance (MDR). Cells displaying MDR become resistant both to anthracyclines and to other agents such as the vinca alkaloids, actinomycin D and the epipodophyllotoxins (Plosker and Faulds, 1993; Berman and McBride, 1992). Typically, MDR cells take up less drug than sensitive equivalents. This phenomenon may result from plasma membrane drug efflux pumps such as P-glycoprotein (P-gp, Moscow et al., 1993), although the mechanism remains controversial (Roepe, 1992). Multidrug resistance-associated protein (MRP, Zaman et al., 1994) and lung resistance-related protein (LRP, Scheffer et al., 1995) have also been recently described as mediating MDR.

Because anthracyclines fluoresce, it is possible to visualise their intracellular distribution. Viable sensitive and MDR cells appear to sequester conventional anthracyclines differently (Coley et al., 1993; Gervasoni et al., 1991; Duffy et al., 1996). Sensitive cells display nuclear drug fluorescence, whereas MDR cells show predominantly cytoplasmic fluorescence. Interestingly, MDR-reversing agents such as verapamil cause the intracellular distribution of these drugs in MDR cells to revert to the sensitive pattern (Coley et al., 1993).

Idarubicin is a relatively new anthracycline. Of greater lipophilicity than earlier derivatives, it appears more effective than its predecessors, especially in the treatment of tumours resistant to these agents (Berman et al., 1992). We have studied the distribution of idarubicin fluorescence in sensitive and P-glycoprotein-expressing MDR sublines of the MGHU-1 bladder cancer cell line (Floyd et al., 1990).

Materials and methods
Sensitive and MDR clones of the MGHU-1 bladder cancer cell line were obtained from the Institute of Urology, UCL. The resistant subline was produced by continuous exposure to doxorubicin and has been shown to express P-gp (Floyd et al., 1990). Studies using JSB1 and MRP have demonstrated high expression of P-gp in the MDR subline, but no overexpression of MRP (MC Loizidou, personal communication). The cells were cultured using Dulbecco’s modified Eagle medium (DMEM), 10% fetal calf serum (FCS) and antibiotics at 37°C in humidified 5% carbon dioxide in air. Subculture was achieved using trypsin-EDTA. Anthracycline (Pharmacia) stock solutions, (1 mg ml⁻¹ Hanks’ balanced salt solution) were frozen at −20°C.

Confocal microscopy
Sensitive and resistant MGH-U1 cells were seeded into 60 mm Petri dishes and reincubated overnight, allowing the cells to adhere. Two hours before microscopy, the medium was changed to HEPES-buffered DMEM with 10 μg ml⁻¹ of anthracycline with or without verapamil 25 μg ml⁻¹. Cell viability after confocal microscopy was confirmed by trypan blue exclusion (0.02% w/v).

Confocal microscopy was performed using the Leica TCS 4D system, the fibre optic laser emitting at 488 nm. Cells were imaged in the incubation medium using a ×50 water immersion lens. Consistent images were obtained on three separate occasions using identical incubation conditions. Pinhole and electronic variables were kept constant throughout.

MTT cytotoxicity studies
Cytotoxicity experiments were performed using the MTT assay (Freshney, 1994). Cells were seeded in 96-well microtitre plates and exposed to drug at 37°C for 1 h on the following day. Five days later, the plates were incubated with MTT (0.2 mg ml⁻¹, 250 μl per well) for 4 h, treated with dimethyl sulfoxide (DMSO) and viable biomass determined on a Dynatech MR 5000 plate reader.

Spectrofluorimetry studies
Spectrofluorimetry experiments were carried out at drug concentrations of 10 μg ml⁻¹ using a Perkins-Elmer LS-5B scanning spectrofluorimeter.

Results
Confocal microscopy uses dual-pinhole optics and raster pattern scanning to produce high definition, fluorescence-based images, enabling intact, viable cells to be visualised as a series of slices. Figure 1a shows the typical, predominantly nuclear epirubicin distribution in sensitive MGHU-1 cells. In contrast, Figure 1b shows punctate cytoplasmic and perinuclear epirubicin distribution in the MDR MGHU-1 cells. These results correspond with published results using other conventional anthracyclines such as daunorubicin or doxorubicin (Coley et al., 1993; Gervasoni et al., 1991).
Figure 2a shows idarubicin distribution in sensitive MGHU-1 cells. Unlike other anthracyclines in sensitive cells, idarubicin fluorescence appears predominantly perinuclear and cytoplasmic. In some cells an area of intense cytoplasmic drug fluorescence is visible, possibly representing the Golgi apparatus. There is relatively little nuclear drug fluorescence.

Figure 2b demonstrates idarubicin distribution in MGHU-1-resistant cells. Although this specimen shows reduced idarubicin fluorescence, drug distribution remains similar to the sensitive cells. Addition of 25 μg ml⁻¹ of verapamil to the idarubicin solution increases drug fluorescence in the resistant cells, but appears to make no difference to the distribution of idarubicin fluorescence in either sensitive (Figure 3a) or resistant (Figure 3b) MGHU-1 cells. MTT cytotoxicity studies confirm the P-gp-expressing subline to 100-fold more resistant to idarubicin (Figure 4). Addition of 25 μg ml⁻¹ of verapamil reduces this resistance by a factor of ten.

DNA-mediated fluorescence quenching demonstrates that idarubicin fluoresces more strongly in free solution than the related anthracyclines epirubicin and doxorubicin (Figure 5).

Between DNA concentrations of 0.02–0.06 mg ml⁻¹, idarubicin demonstrates a greater degree of fluorescence quenching. At greater concentrations, however, the pattern of quenching appears the same.

**Discussion**

These results contrast with studies performed on conventional anthracyclines by ourselves and others. Using doxoauno- and epirubicin, nuclear drug fluorescence has been associated with sensitivity, and cytoplasmic fluorescence with resistance (Coley et al., 1993; Gervasoni et al., 1991). Additionally, the morpholinyl-substituted analogue of doxorubicin (MR-DOX) is known to retain activity in MDR cells, and high levels of nuclear MR-DOX fluorescence in MDR cell lines have been demonstrated (Coley et al., 1993).

Although the precise antineoplastic mechanism of action of anthracyclines is still debated, current evidence suggests that these agents intercalate DNA. They may stabilise the topoisomerase–DNA cleavable complex or inhibit DNA helicase activity, thereby reducing replication and transcrip-
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Figure 3 Confocal micrograph of (a) sensitive and (b) resistant MGH-U1 cells incubated for 2h in 10µg ml⁻¹ idarubicin and 25 µg ml⁻¹ verapamil, ×50 water immersion objective. Grey-scale image proportional to fluorescence intensity.

Our own MTT cytotoxicity studies confirm that addition of verapamil to idarubicin preparations increases idarubicin cytotoxicity in the MDR MGH-U1 cell line (Figure 5). However, confocal microscopy demonstrates that the addition of verapamil to idarubicin does not restore nuclear drug fluorescence in either the parental or MDR cell line, suggesting that substantial nuclear drug presence may not be a prerequisite for effective anthracycline cytotoxicity in sensitive cells, or for overcoming MDR.

Considerable work remains to be done, both on the putative mechanism of action of anthracyclines, and on the fundamentals of MDR and MDR reversal. We believe that our results are relevant to the continuing study of these mechanisms and that it is premature to assume that nuclear drug fluorescence necessarily correlates with cytotoxicity.

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