ORIGINAL ARTICLE

Furosemide reverses multidrug resistance status in bladder cancer cells in vitro

A G Speers, B A Lwaleed, J M Featherstone, B J Sallis, A J Cooper

J Clin Pathol 2006;59:912–915. doi: 10.1136/jcp.2005.033100

See end of article for authors' affiliations

Correspondence to:
B A Lwaleed, Department
of Urology, Central block,
Level E, West Wing,
Mailpoint 67,
Southampton University
Hospitals NHS Trust,
Tremona Road,
Southampton, SO16 6YD,
UK; bashir@soton.ac.uk

Accepted for publication
8 December 2005
Published Online First
23 March 2006

Background: Multidrug resistance (MDR) has a potentially serious influence on cancer treatment and should be taken into consideration in the design and application of therapeutic regimens. It is mediated through the activity of cellular pumps.

Aim: To investigate whether furosemide, itself a pump-blocker, reverses MDR in an in vitro model.

Materials and methods: An MDR bladder cancer cell line (MGH-u 1R) and its parental (drug sensitive) clone were exposed to epirubicin and furosemide, with the concentration of one drug fixed and that of the other serially diluted in a 96-well plate format. Both drugs formed the variable component in separate experiments. After a 1-h exposure, the cells were washed and replenished with fresh medium. To examine the toxicity of epirubicin and furosemide separately and in combination, monotetrazolium-based assays were carried out. Intracellular epirubicin distribution was assessed by confocal microscopy as a second index of resistance status after in vitro exposure.

Results: MGH-u 1R cells incubated with furosemide showed distribution of drug similar to that in the parental cells (MGH-u 1 sensitive). Controls (without furosemide) continued to show a resistant pattern of fluorescence. In cytotoxicity assays furosemide appeared substantially non-toxic. Resistant cells in the toxicity titration experiments showed increased resistance to levels of furosemide over 500 µg/ml. Parental cells were made only marginally more sensitive against increased background toxicity.

Conclusion: Furosemide is effective in reversing MDR status in bladder cancer cell lines in vitro. It may also have an increment of intrinsic cytotoxicity, but only at higher concentrations. We propose a potential for further investigation of furosemide as an adjunct to chemotherapy for superficial bladder cancer.

Bladder cancer is common in the UK, with an estimated incidence of 12 000 new cases per year. Approximately 90% of these are transitional cell carcinoma in origin,¹ with the remaining 10% being mainly a mixture of adenocarcinoma and squamous cell carcinoma. Of the population with transitional cell carcinoma, 60–75% of the cancers are deemed to be superficial in nature, amenable to “curative” surgical resection. Patients undergoing resection for superficial disease have only a 10–15% chance of developing muscle invasive disease, but 50–80% of this group will have superficial tumour recurrence.² Tolley *et al*³ have shown that recurrence rates can be reduced by 34–50% with adjuvant intravesical chemotherapy at the time of primary resection. However, a subgroup of patients who receive chemotherapy develop multidrug resistance (MDR) to chemotherapeutic agents.

MDR is usually associated with decreased intracellular concentrations of cytostatic drugs. The mechanism of this is multifactorial, but of greatest importance is the overexpression of P-glycoprotein (P-gP). The breast cancer resistance protein and MDR-related protein may also be up regulated.⁴ P-gP expression has been observed in urothelial cancer cells before chemotherapy.⁵ Development of resistance may be due to cell selection or up regulation of P-gP gene expression. P-gP is a 170 kDa plasma membrane glycoprotein with six transmembrane domains and two adenosine triphosphate (ATP)-binding sites,⁶ functioning as an ATP-dependent efflux pump. Its expression occurs naturally in other tissues; these include hepatocytes, proximal convoluted tubule cells and bowel mucosa.

MDR can be reversed by mechanisms aimed at inhibiting P-gP function. Agents or actions that have been shown to reverse MDR include calcium channel blockers,⁷ Estramustine,⁸ altering intracellular pH,⁹ H1-blockers¹⁰ and

steroids.¹¹ Furosemide is a loop diuretic, which functions by inhibiting the mechanism of the sodium or potassium or chloride pump in the ascending limb of the loop of Henle. It is an ATP-dependent pump. The fact that furosemide's main use clinically is to stop sodium re-absorption in the ascending limb of the loop of Henle by blocking pump function suggests that it may have a similar effect on the P-gP pump. Here, using well-established in vitro models,^{8–12–13} we examine whether furosemide reverses MDR uptake of the anthracycline epirubicin, which is a member of the class of MDR cross-reacting agents that also includes mitomycin C.¹⁴

MATERIALS AND METHODS

Cells

The adriamycin-induced MDR variant of the bladder cancer cell line (MGH-u 1R)¹⁵ and its parental clone were grown in adherent monolayer culture in Dulbecco's modified Eagle's medium (Sigma-Aldrich, Poole, UK) supplemented with 10% fetal calf serum, penicillin, streptomycin and glutamine. The 37°C incubator was gassed with 5% CO₂ in air at 100% humidity.

Intracellular drug localisation

Experimental cells were seeded into 60-mm-diameter culture-grade petri dishes. Epirubicin (20 µg/ml) was added to the medium and the plates were incubated for 1 h at 37°C and 5% CO₂. At 1 h, epirubicin is taken up to about 80% of the maximum¹⁶; this is also the relevant period for intravesical instillation. Subsequently, the medium was decanted and the cells were washed in phosphate-buffered

Abbreviations: ATP, adenosine triphosphate; MDR, multidrug resistance; MGH-u 1R, MDR bladder cancer cell line; P-gp, P-glycoprotein

saline. The medium was replaced by phosphate-buffered saline and the intracellular fluorescence pattern of epirubicin in the treated cells was viewed using a Zeiss LSM 510 confocal microscope visualising drug autofluorescence excited by 488 nm light. To assess MDR reversal, cells were incubated with furosemide, at a range of doses from 1 to 20 mg/ml, and epirubicin 20 µg/ml for 1 h. The intracellular anthracycline distribution was assessed by confocal microscopy as above. Cell adhesion and morphology in these preparations remained good. Formal viability testing (trypan blue and fluorescein diacetate tests) was carried out at furosemide concentrations up to 20 mg/ml for 1 h of incubation. Cells so treated also continued to grow normally when returned to the incubator.

Cytotoxicity assay

MGH-u 1R cells were seeded into 96-well microtitre plates and left to settle for 24 h. In experiments in which epirubicin was titrated, the columns of wells were filled with medium containing either 5 or 1 mg/ml furosemide with serial doubling dilution of epirubicin along columns 1–10 (100 µg/ml down to 0.195 µg/ml). Columns 11 and 12 were used for 100% growth and blank controls. Parallel plates were treated with epirubicin using the same dilution pattern but without furosemide. Where furosemide was titrated the plates were used in “portrait” format: the upper half was used for resistant cells and the lower half for sensitive cells. Furosemide was titrated across seven columns, starting with a jump from 5 to 1 mg/ml, with doubling dilutions thereafter.

Figure 1 Sensitive and resistance bladder cancer cell lines incubated with epirubicin alone or in combination with furosemide as viewed by confocal microscopy: (A) multidrug resistance bladder cancer cell line (MGH-u 1)-sensitive cells and (B) MGH-u 1R cells. The MGH-u 1R cells were incubated with epirubicin and a range of furosemide doses for 1 h, showing a sensitive pattern of epirubicin uptake characterised by pronounced nuclear uptake of epirubicin and increased concentrations of fluorescence: (C) 1 mg/ml, (D) 5 mg/ml, (E) 10 mg/ml and (F) 20 mg/ml. Fluorescence is superimposed on differential interference contrast images, to show morphology.

The eighth column was left as an epirubicin-only control. Plates were incubated for 1 h at 37°C and 5% CO₂, washed with phosphate-buffered saline and re-filled with 150 µl of fresh culture medium. After incubation for a further 48 h, the culture medium was thrown off and 50 µl of monotezazolium added for 2 h incubation. Longer incubation times were not practical as the control wells became post-confluent. The working monotezazolium solution comprised a 1:3 v/v mixture with culture medium of 5 mg/ml aqueous stock solution. After incubation, the supernatant was thrown off and the blue formazan crystals that had formed in the viable cells were solubilised by the addition of 150 µl of dimethyl sulphoxide into each well. The plate was shaken to dissolve the formazan fully and the amount present was determined on a spectrophotometric plate reader at 570 nm. Results are expressed as percentage of growth inhibition or 100 minus residual viable biomass. In previous publications, our group has tended to present residual viable biomass, which we now believe is a slightly counter-intuitive method of presentation.

RESULTS

Intracellular drug localisation

Sensitive bladder cancer cell line MGH-u 1 incubated with epirubicin alone showed a pronounced nuclear uptake of epirubicin and high levels of fluorescence (fig 1A) compared with a typical distribution of epirubicin uptake by the MGH-u 1R cells (fig 1B). The pattern of epirubicin fluorescence after incubating MGH-u 1R cells with furosemide and epirubicin showed reversal to a sensitive phenotype. There is a clearly marked nuclear epirubicin uptake characteristic of the MGH-u 1 sensitive cells at 1, 5, 10 and 20 mg/ml of furosemide (fig 1C–F). A time series study showed that furosemide-induced reversal of resistance, as seen by nuclear uptake, occurred typically between 30 and 45 min after exposure to furosemide.

Cytotoxicity

Results of monotezazolium assay from triplicate plates are illustrated in figs 2 and 3. An increase in cytotoxicity was found when epirubicin dilutions were coincubated with furosemide, compared with controls (fig 2). The results are suggestive of increased cytotoxicity in resistant cells but leave room for speculation on how much of the apparent effect can be ascribed to the native toxicity of furosemide. Titrating furosemide, shown as a bar chart owing to the uneven x-axis scale, shows furosemide to enhance epirubicin in a supra-additive fashion toxicity in resistant cells down to 1 or perhaps 0.5 mg/ml. Thereafter, it hovers around the same toxicity as the epirubicin-only control (fig 2). The high kill rate of 50 µg/ml epirubicin on sensitive (parental) cells is increased relatively evenly and to completion by furosemide

Figure 2 Furosemide titrations using multidrug resistance (MDR) bladder cancer cell line (MGH-u1) cells plotted as change in growth inhibition (toxicity) compared with an epirubicin-only control. The epirubicin concentration was 50 µg/ml throughout the plate.

except at the highest concentration (fig 2). Parental cells respond to epirubicin alone, with a sigmoid curve falling steeply from 90 to 4% toxicity between 6 and 1 µg/ml (fig 2). Furosemide is minimally toxic, even with continued incubation, to 0.2 mg/ml (10% or less); at 0.5 mg it rises to 15% and achieves 34% at 5 mg/ml (fig 4).

DISCUSSION

Adjuvant chemotherapy is an important element in treating superficial bladder cancer,¹⁷ with a reduction of about 34–50% in recurrence rates in those receiving treatment. MDR is a significant complication in delivering effective chemotherapy, with up to 60% of cancers expressing markers of MDR in at least a subpopulation of cells at presentation.^{5, 18}

Epirubicin is a cytotoxic semisynthetic derivative of the anthracycline doxorubicin family used in the treatment of a wide range of malignancies, including those of the breast, bladder and lung. It is a cell cycle-active cytotoxic agent without phase specificity. Its mode of action is not fully understood, but several mechanisms have been identified. The main mechanism is the intercalation of DNA strands via topoisomerase II activity, which prevents RNA and DNA production leading to cell death. Free radical production by microsomal enzymes such as the reduced form of nicotinamide-adenine dinucleotide phosphate and cytochrome P450 reductase reduces anthracyclines to semiquinone-free radicals, enhancing cytotoxicity. It has also been suggested that epirubicin can cause injury through direct effects on cell membranes by impairing calcium and sodium transport.¹⁹ Thus, for successful anthracycline-based adjuvant intravesical chemotherapy, both intracellular and nuclear uptake are essential. This translocation is lacking in MDR cells. To date, a variety of drugs have been shown to reverse the MDR pattern, including calcium antagonists, steroids and immunosuppressants.^{7–11} They act by several mechanisms, but generally all aim at interrupting the ATP-driven efflux pump, hence permitting intracellular accumulation of cytotoxic agents.

Furosemide is a loop diuretic, which, in the ascending limb of the loop of Henle, works by actively competing with chloride ions on the sodium–potassium–chlorine cotransport mechanism.²⁰ This prevents sodium from being re-absorbed in the counter-current mechanism, thus leading to an

Figure 3 Epirubicin titrations using multidrug resistance (MDR) bladder cancer cell line (MGH-u1) cells plotted as percentage of growth inhibition. The toxicity curve for the parental clone is presented for reference purposes, with an IC₅₀ of approximately 2 µg/ml epirubicin. The comparable IC₅₀ for untreated MGH-u1R cells was attained at around 50 µg/ml. Coincubation with furosemide at 1.0 mg/ml (MDR F1) raised the toxicity at lower epirubicin concentrations; 5.0 mg/ml furosemide (MDR F5) gave a flat response to epirubicin at about 60% toxicity relative to control growth.

Figure 4 Furosemide titrated in the absence of epirubicin. The x axis has a discontinuity of scale, the first two points representing a fivefold dilution and the rest doubling dilutions.

increase in the osmolarity of the tubular fluid, promoting diuresis. By reducing sodium reabsorption, diuretics also reduce the intramedullary sodium concentration, hence reducing the osmotic gradient between the tubule lumen and the renal medulla and diminishing the effect of the counter-current multiplier and promoting further diuresis. In addition to reducing sodium re-absorption, loop diuretics also promote loss of other cations, which mainly include potassium, calcium and magnesium. Furosemide is a widely used, safe drug that is secreted by the kidneys and concentrated in the urine. Experimental drug doses required to produce reversal of MDR *in vitro* are low by clinical standards. The bladder is an ideal target for MDR reversing agents, as they can be given in the high doses often required to produce locally cellular effect, with no risk of systemic toxicity. In this context, furosemide has the benefit of being a familiar and safe drug with few side effects.

The intracellular pattern of anthracycline distribution is well documented across several bladder cancer cell lines.^{13 15 17 21 22} Sensitive cells have predominantly nuclear fluorescence, whereas resistant cells have only a weak cytoplasmic fluorescence. In this study, the distribution patterns of fluorescence were used as a model on which reversal of resistance by exposure to furosemide was examined. Reversal of resistance by furosemide was shown to occur in 45 min after administration. This is too quick for furosemide toxicity to cause nuclear uptake, a contention supported by the good morphology of the cells examined. In cytotoxicity experiments, with a dose of 5 mg/ml of furosemide the percentage of cell death remained constant across the columns even though the dose of epirubicin exposure decreased by more than a hundred fold. This is probably because in sensitising the resistant cells lower anthracycline doses were required to enable access to the nucleus and eventually cause cell death. A range of effective drug concentrations will exist through a solid tumour papilla. It is potentially advantageous that the furosemide effect operates at low concentrations.

The exact mechanism by which furosemide may reverse MDR through sodium transport modulation remains unclear. Several possible explanations, however, do exist. Furosemide, by antagonising the sodium–potassium–chlorine pump, interferes with sodium pump function on the cell membrane, changing the mechanism of cellular homeostasis, which may in turn increase the cellular permeability to cytotoxic agents by affecting the P-gp function. Loop diuretics are known to decrease the plasma concentration of magnesium. This cation is essential for the binding of ATP to P-gp for the provision of energy, allowing pump function.⁶ Furosemide may also reduce the intracellular magnesium concentration, thus preventing ATP binding and energy utilisation. Further work is required to consider these points.

In conclusion, furosemide reverses the MDR status in the MGH-u 1R bladder cancer cell line. The fact that clinically, 50% of an oral dose of furosemide is excreted unchanged in the urine and 20% is excreted as furosemide glucuronide adds further intrigue regarding its potential as an agent for prolonging MDR reversal in cells that have accumulated drug, after the free drug is washed out. We therefore propose that furosemide can be a useful adjunct in the chemotherapeutic management of vesicular malignancy. As a well-tolerated and much-used agent in urological practice, its acceptance in a novel role should be that much easier.

Authors' affiliations

A G Speers, B J Sallis, A J Cooper, Department of Biomedical Sciences, University of Portsmouth, Portsmouth, UK

B A Lwaleed, J M Featherstone, Department of Urology, Southampton University Hospitals NHS Trust, Southampton, UK

Competing interests: None declared.

REFERENCES

- Burnand K, Young A. *The new Aird's companion in surgical studies*, 2nd edn. London: Churchill Livingstone, 1998:1081–9.
- Richie JP. Intravesical chemotherapy. Treatment selection, techniques, and results. *Urol Clin North Am* 1992;**19**:521–7.
- Tolley DA, Parmar MK, Grigor KM, *et al*. The effect of intravesical mitomycin C on recurrence of newly diagnosed superficial bladder cancer: a further report with 7 years of follow up. *J Urol* 1996;**155**:1233–8.
- Scheper RJ, Scheffer GL, Flens MJ. Transporter molecules in multidrug resistance. *Cytotechnology* 1996;**19**:187–90.
- Nakagawa M, Emoto A, Nasu N, *et al*. Clinical significance of multi-drug resistance associated protein and P-glycoprotein in patients with bladder cancer. *J Urol* 1997;**157**:1260–5.
- Varadi A, Szakacs G, Bakos E, *et al*. P glycoprotein and the mechanism of multidrug resistance. In: *Mechanism of drug resistance in epilepsy: lessons from oncology*. Novartis Foundation Symposium. Chichester: Wiley, 2002:54–68.
- Weaver JL, Szabo G Jr, Pine PS, *et al*. The effect of ion channel blockers, immunosuppressive agents, and other drugs on the activity of the multi-drug transporter. *Int J Cancer* 1993;**54**:456–61.
- Jennings AM, Solomon LZ, Sharpe P, *et al*. Estramustine reversal of resistance to intravesical epirubicin chemotherapy. *Eur Urol* 1999;**35**:327–35.
- Roepe PD. pH and multidrug resistance. In: *The tumour microenvironment: causes and consequences of hypoxia and acidity*. Novartis Foundation Symposium. Chichester: Wiley, 2001:232–50.
- Ibrahim S, Peggins J, Knapton A, *et al*. Influence of beta-adrenergic antagonists, H1-receptor blockers, analgesics, diuretics, and quinolone antibiotics on the cellular accumulation of the anticancer drug, daunorubicin: P-glycoprotein modulation. *Anticancer Res* 2001;**21**:847–56.
- Lewin J, Cooper A, Birch B. Progesterone: a novel adjunct to intravesical chemotherapy. *BJU Int* 2002;**90**:736–41.
- Duffy PM, Hayes MC, Cooper A, *et al*. Confocal microscopy of idarubicin localisation in sensitive and multidrug-resistant bladder cancer cell lines. *Br J Cancer* 1996;**74**:906–9.
- Duffy PM, Hayes MC, Gattrell SK, *et al*. Determination and reversal of resistance to epirubicin intravesical chemotherapy. A confocal imaging study. *Br J Urol* 1996;**77**:824–9.
- Hayes MC, Birch BR, Cooper AJ, *et al*. Cellular resistance to mitomycin C is associated with overexpression of MDR-1 in a urothelial cancer cell line (MGH-U1). *BJU Int* 2001;**87**:245–50.
- McGovern F, Kachel T, Vijan S, *et al*. Establishment and characterization of a doxorubicin-resistant human bladder cancer cell line (MGH-U1R). *J Urol* 1988;**140**:410–4.
- Duffy PM, Hayes MC, Cooper A, *et al*. Determination and reversal of resistance to epirubicin intravesical chemotherapy. A flow cytometric model. *Br J Urol* 1996;**77**:819–23.
- Knuchel R, Hofstadter F, Jenkins WE, *et al*. Sensitivities of monolayers and spheroids of the human bladder cancer cell line MGH-U1 to the drugs used for intravesical chemotherapy. *Cancer Res* 1989;**49**:1397–401.
- Cooper AJ, Hayes MC, Duffy PM, *et al*. Multidrug resistance evaluation by confocal microscopy in primary urothelial cancer explant colonies. *Cytotechnology* 1996;**19**:181–6.
- Plosker GL, Faulds D. Epirubicin. A review of its pharmacodynamic and pharmacokinetic properties, and therapeutic use in cancer chemotherapy. *Drugs* 1993;**45**:788–856.
- Laurence DR, Bennett PN. Kidney and urinary tract. *Clinical pharmacology*. 7th edn. London: Churchill Livingstone, 1992:459–69.
- Davies CL, Duffy PM, MacRobert AJ, *et al*. Localization of anthracycline accumulation in sensitive and resistant urothelial tumor cell lines. *Cancer Detect Prev* 1996;**20**:625–33.
- Featherstone JM, Speers AG, Lwaleed BA, *et al*. The nuclear membrane in multidrug resistance: microinjection of epirubicin into bladder cancer cell lines. *BJU Int* 2005;**95**:1091–8.