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Relative cytotoxicities of Adriamycin and epirubicin in combination with lonidamine against human bladder cancer cell lines

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Abstract We have used a panel of bladder cancer cell lines to compare the toxicities of Adriamycin and epirubicin, two drugs used intravesically to treat superficial transitional cell cancer (TCC) of the bladder, alone and in combination with lonidamine, an agent known to be active against anthracycline-resistant disease. Comparing concentrations reducing colony-forming ability by 50%, epirubicin and Adriamycin were similar in their cytotoxicities, although epirubicin was more potent against every line except an Adriamycin-resistant subline. Combinations of the two drugs with a non-cytotoxic concentration (1 μg/ml) of lonidamine were tested using the Adriamycin-resistant subline MGH-U1R and its sensitive parental line MGH-U1. The addition of lonidamine caused a two-fold increase in the sensitivity of the resistant subline to both drugs, while having no effect on the sensitivity of the parental line. The data indicate that this combination might be of value in anthracycline-resistant disease.

Key words Adriamycin · Epirubicin · Lonidamine Multidrug resistance · Human transitional carcinoma cell lines · In vitro sensitivity

Intravesical chemotherapy is used as a prophylactic treatment to reduce the rate of recurrence of superficial bladder cancer (pTa and pT1), and to treat tumours that are too widespread for surgical management [33]. The drugs are instilled as single agents in aqueous solution for a limited period, usually between 1 and 2 h. Adriamycin, epirubicin, mitomycin-C and Thiotepa are the drugs most frequently used, and there is little difference in their activity for the treatment of superficial bladder cancer [13]. When used as definitive treatment, these agents induce a complete response in approximately one-third of patients, a partial response in one-third and have no effect in approximately one-third [32].

Resistance to anthracyclines such as Adriamycin and epirubicin can be mediated through amplification of p-glycoprotein (p170) [24]. This transmembrane protein is an energy-dependent efflux pump that removes drug from cells. Resistance caused by overexpression of p170 is termed multidrug resistance (MDR), because it is associated with cross-resistance to a number of other unrelated cytotoxic drugs [25].

Reversal of multidrug resistance is a major goal in experimental cancer research [25, 43]. Using cell lines [9] and experimental tumours overexpressing p170 [38], a large number of agents have been shown to interact with p170 and partly reverse drug resistance [51]. Lonidamine (LND) is one agent that has been shown to modify the sensivity of Adriamycin-resistant cells [7]. It is a derivative of indazole carboxylic acid that causes mitochondrial damage, inhibiting both oxygen consumption and glycolysis [10, 14–17], but does not affect cell division [23]. Its antitumour activity is thought to be related to inhibition of repair of potentially lethal damage [22]. It has limited activity against experimental tumours as a single agent [6, 48], and in the clinic has some activity against breast [1, 41], prostate [12] and non-small cell lung cancer [29, 46], but not against head and neck cancer [3]. However, in experimental models it has greater activity when combined with radiotherapy [8, 26, 28], cytotoxic drugs [49, 53] and hyperthermia [27, 48]. In combination with hyperfractionated radiotherapy it increased the proportion of long-term disease-free survivors with head and neck cancer [34].

Because Adriamycin and epirubicin are used as single agents in the treatment of superficial bladder cancer, and because drug resistance is the major limitation to their ef-

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Table 1 Some characteristics of the bladder cancer cell lines used in this study

Cell line	Passage nos	Biopsy origin	Prior treatment of patient	References
MGH-U1 (T24)	106–116	TCC bladder recurrence	None	Bubenik et al 1973 [5]
MGH-U1R	22–32	MGH-U1		McGovern et al 1988 [37]
RT112	41–51	TCC bladder primary	None	Masters et al 1986 [36]
HT 1376	64–74	TCC bladder primary	None	Rasheed et al 1977 [44]
RT 4	78–88	TCC bladder recurrence	Gold grains	Rigby & Franks 1970 [45]
VM-CUB-III	22–32	TCC bladder primary	Not known	Williams 1980 [52]

fectiveness, the addition of lonidamine to intravesical chemotherapy might increase the therapeutic value. In this study, we demonstrate in vitro that, comparing the sensitivities of five untreated cell lines, epirubicin is slightly more potent than Adriamycin. An Adriamycin-resistant bladder cancer cell line overexpressing p170 was more sensitive to lonidamine than its parental line, and LND partially reversed resistance to both drugs.

Materials and methods

Cell lines and culture conditions

Details of the passage numbers, the origin of each of the cell lines and any treatment received before the tumour was biopsied are shown in Table 1. The cells were maintained under identical culture conditions as monolayers in 25-cm flasks (Nunc Gibco, Paisley, Scotland) in either RPMI 1640 or MEM alpha medium (Gibco) supplemented with 5% heat-inactivated fetal calf serum (FCS) and 2 mM L-glutamine at 36.5 °C in a humidified atmosphere of 5% CO_2 in air. The cells were routinely subcultured using a mixture of 0.05% trypsin (Difco, London) in 0.016% Versene [ethylenediaminetetra-acetic acid (EDTA) BDH Chemicals, Poole, UK]. The cell lines were used over a restricted range of ten in vitro passages to minimise changes occurring as a result of long-term culture.

Cytotoxic drugs

Both Adriamycin and epirubicin (Pharmacia) were supplied as sterile clinical preparations in injectable form, and were stored at 4 °C. The appropriate concentrations of drugs were diluted in cell culture medium immediately before use. The concentrations used for the experiments ranged from 5 to 25 ng/ml for the sensitive cell lines and from 100 to 7000 ng/ml for the resistant cell lines. Dimethylsulphoxide (Sigma) was used in the control dishes at a concentration of 1% for the experiments including lonidamine. The lonidamine was provided by the Angelini Research institute, Rome, and was dissolved in dimethylsulphoxide (DMSO) to give a final concentration of 1% DMSO in culture medium.

Drug sensitivity experiments

To determine the sensitivity of the cell lines to epirubicin, Adriamycin and lonidamine alone, colony-forming abilities were determined as follows. For monolayer cultures, approximately 1000 cells were plated per 5-cm Petri dish and incubated for 24 h in 5% CO_2 in air to allow exponential cell growth to resume. A 50-µl aliquot of the drug dissolved in medium or DMSO (lonidamine) of the desired concentration was added to each 5-cm Petri dish containing 4950 µl medium. The sensitivity of the cell lines MGH-U1 and MGH-U1R to Adriamycin or epirubicin in the presence and absence of lonidamine at a concentration of 1 µg/ml were also determined. Control samples were run with medium or 1% DMSO alone. Following addition of the drug, the Petri dishes were returned to the incubator. After 10–14 days the plates were fixed in methanol and stained with Giemsa. Colonies of more than 50 cells were counted using a binocular dissecting microscope. IC_{50} values were determined from individual experiments.

Results

The dose responses of the five bladder cancer cell lines to Adriamycin and epirubicin are shown in Figs. 1 and 2. Comparing the concentrations of each drug required to reduce colony-forming ability by 50% (IC_{50}), epirubicin was the more potent agent against every cell line (Table 2). However, the differences in sensitivity were relatively small, with IC_{50} ratios for Adriamycin/epirubicin ranging from 1.1 for MGH-U1 and VM-CUB-III to 1.9 for HT1376 (Table 2). For Adriamycin there was an approximately fourfold range of IC_{50}s from 4.6 to 20.1 ng/ml, and similarly for epirubicin from 4.3 to 17.1 ng/ml. These differences are small compared with the difference between the sensitive line MGH-U1 and its Adriamycin-resistant derivative MGH-U1R.

The drug-resistant subline of MGH-U1 was 409 times more resistant to Adriamycin and 663 times more resistant to epirubicin than the parental line, comparing IC_{50}s. However, in this case the relative potency of the two drugs was reversed, and over 50% more epirubicin than Adriamycin was required to reduce the colony-forming ability by 50% (Table 2).

Table 2 Relative cytotoxicities of Adriamycin and epirubicin against five human bladder cancer cell lines and an Adriamycin-resistant subline, showing the mean and standard errors, from a minimum of three separate experiments, of the concentration of each drug required to reduce colony-forming ability by 50%

Cell line	Adriamycin IC_{50} values (ng/ml)	Epirubicin IC_{50} values (ng/ml)	Ratio of Adriamycin/ epirubicin IC_{50}s
HT1376	16.7 ± 2.3	8.6 ± 1.9	1.9
VM-CUB-III	19.1 ± 1.0	17.1 ± 1.6	1.1
RT4	20.1 ± 1.2	12.3 ± 0.7	1.6
RT112	10.3 ± 2.0	8.3 ± 1.4	1.2
MGH-U1	4.6 ± 0.2	4.3 ± 0.5	1.1
MGH-U1R	1882 ± 167	2850 ± 278	0.7

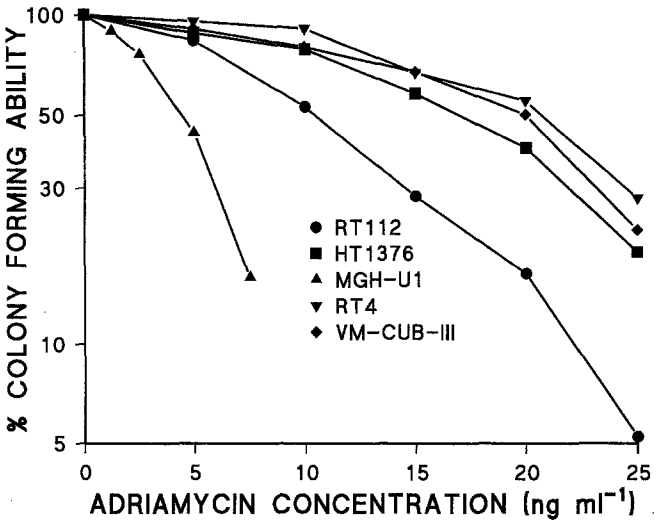

Fig. 1 Dose-response curves of five human bladder cancer cell lines to increasing concentrations of Adriamycin. Each experiment was repeated at least 3 times. Standard errors are not included in this figure to aid clarity, but are shown in Table 2

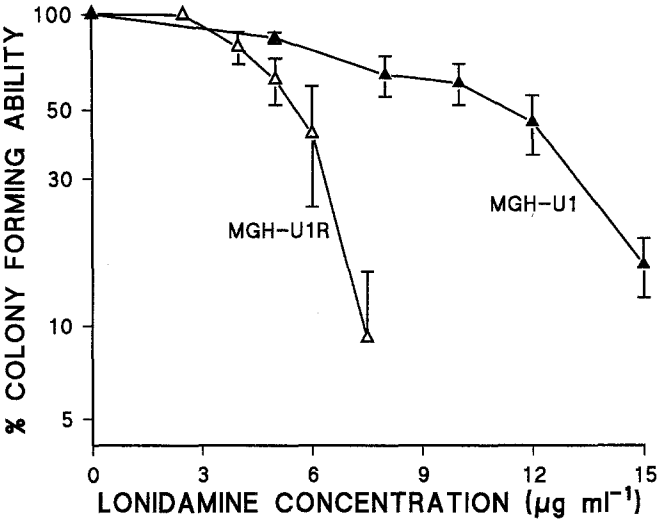

Fig. 3 Sensitivities of the parental line MGH-U1 and its Adriamycin-resistant derivative, MGH-U1R, to increasing concentrations of lonidamine. The values are derived from a minimum of three separate experiments, and standard error bars are included

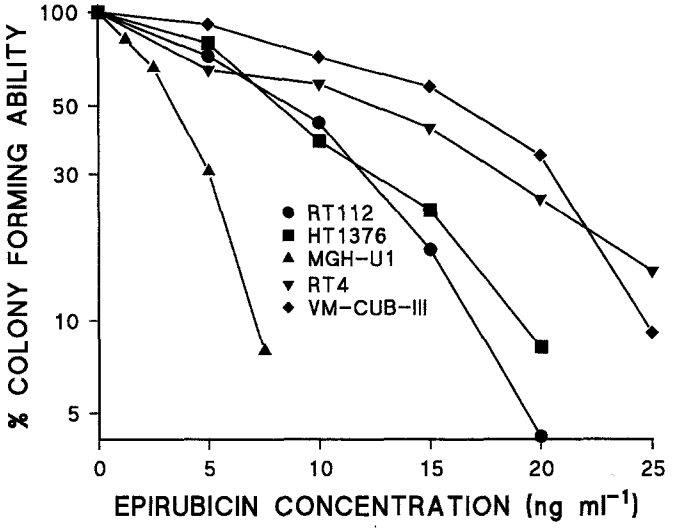

Fig. 2 Dose-response curves of five human bladder cancer cell lines to increasing concentrations of epirubicin. Each experiment was repeated at least 3 times. Standard errors are not included in this figure to aid clarity, but are shown in Table 2

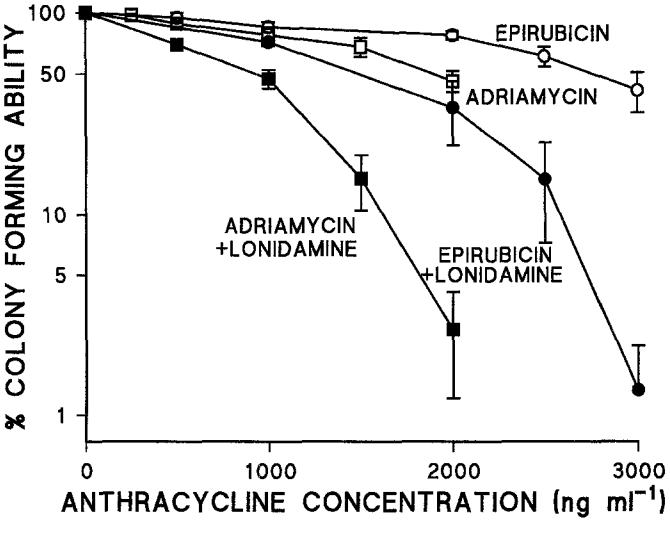

Fig. 4 Sensitivities of the Adriamycin-resistant cell line MGH-U1R to epirubicin and Adriamycin alone and in combination with lonidamine. The values are derived from a minimum of three separate experiments, and standard error bars are included

Lonidamine was cytotoxic at high concentrations to both the parental line MGH-U1 and the resistant subline MGH-U1R (Fig. 3). The resistant line was twofold more sensitive to lonidamine than the parental line (Table 3). For the studies of drug combinations a relatively low concentration of lonidamine, 1 μg/ml, was chosen to exclude any effect of lonidamine alone on cytotoxicity, as this concentration was well below the cytotoxic threshold (Fig. 3). The result of combining Adriamycin or epirubicin with lonidamine is shown in Fig. 4. The sensitivity of the resistant subline was increased twofold to both epirubicin and Adriamycin by the addition of lonidamine (Fig. 4). However, for both drugs the sensitivity of the parental line to each of the chemotherapeutic drugs was unaffected by the addition of lonidamine (Table 3).

Discussion

The panel of cell lines showed a similar ranking in sensitivity to both epirubicin and Adriamycin and the untreated cell lines were between 1.1 and 1.9 times more sensitive to epirubicin. The greater potency of epirubicin can be pre-

Table 3 Effect of adding lonidamine to Adriamycin and epirubicin on the survival of a bladder cancer cell line MGH-U1 and its Adriamycin-resistant subline MGH-U1R, showing mean IC_{50}s and standard errors from a minimum of three separate experiments

Cell line	Lonidamine alone IC_{50} (μg/ml)	Adriamycin alone IC_{50} (ng/ml)	Adriamycin +lonidamine IC_{50} (ng/ml)	Epirubicin alone IC_{50} (ng/ml)	Epirubicin +lonidamine IC_{50} (ng/ml)
MGH-U1	11.0 ± 0.9	4.6 ± 0.2	4.3 ± 0.5	3.5 ± 0.2	3.4 ± 0.4
MGH-U1R	5.5 ± 0.5	1883 ± 167	942 ± 131	2850 ± 278	1591 ± 260

dicted on the basis of the relative pK_as and lipophilicities. Both drugs have the same chemical formula and differ only in the configuration of the hydroxyl group at the 4′ position. In Adriamycin this is in the axial configuration, bringing the OH group close to the NH_2 of the amino sugar ring. The groups interact by hydrogen bonding, thus increasing the ability of the NH_2 group to ionise, in turn raising its pK_a. In contrast the OH group in epirubicin is orientated in the equatorial plane away from the NH_2 group, reducing the interaction, the degree of ionisation and the pK_a. The pK_as of Adriamycin and epirubicin were reported to be 8.34 and 8.08, respectively [11]. Thus, epirubicin has a greater lipophilicity at the same pH, and thus a greater proportion of the drug will be in the non-ionised form and in consequence more drug can enter the cell. The practical effect is that at every pH that we compared the toxicities of epirubicin and Adriamycin against the RT112 bladder cancer cell line, epirubicin was more toxic [21].

The cell line MGH-U1R was resistant to epirubicin and Adriamycin, compared to its parental cell line. It was developed by long-term culture in medium supplemented with Adriamycin. The initial report on the characterisation of MGH-U1R described a ninefold increase in resistance to Adriamycin [37]. This cell line continued to be cultured in medium containing Adriamycin and within 1 year had developed a 50-fold resistance and maintained the same level of resistance after 4 months of growth in drug free media [18].

MGH-U1R is cross resistant to vinblastine, vincristine and etoposide whilst remaining sensitive to a second category of agents which includes methotrexate, cyclophosphamide and bleomycin [18]. Verapamil enhanced the cytotoxic effect of Adriamycin against MGH-U1R [30]. Further evidence for multidrug resistance in bladder cancer has come from work using the cell line UM-UC-6 dox. This is an Adriamycin-resistant subline of UM-UC-6 selected in vitro by exposure to Adriamycin, and is reversibly resistant in the presence of verapamil and has increased p-glycoprotein expression by Western blot analysis [47]. Clinical studies in superficial bladder cancer have shown that intravesical verapamil increases the uptake of epirubicin in both the bladder mucosa and tumour tissue, but not in the peripheral blood [31]. This study showed no demonstrable improvement in response in 35 patients treated with epirubicin combined with intravesical verapamil, compared with 40 patients treated with epirubicin alone. Ablation of superficial bladder cancer was observed with the combination of Adriamycin and verapamil [39]. Six of 18 patients had a complete response and 5 a partial response. These studies have confirmed the safety of intravesical verapamil in combination with anthracyclines.

The experiments described in this report show that lonidamine has a comparable effect to verapamil on the modulation of drug resistance in the cell line MGH-U1R. Studies on the influence of verapamil in the MGH-U1R cell line [30] and the UM-UC-6 Adriamycin-resistant subline [47] indicated that the addition of verapamil increased the cytotoxic effect of Adriamycin in the resistant cell lines by a factor of 2.5 to 3.3, respectively. In our study lonidamine increased the cytotoxic effect of epirubicin and Adriamycin by a factor of two.

The ability of lonidamine to influence the cytotoxic effect of various drugs has been investigated previously using tumour cell lines, including breast [7], ovarian cancer cell lines [49], glioma and oral squamous cell carcinoma cell lines [42] and melanoma cell lines [53]. In the breast cancer cell line MCF7Adr the addition of lonidamine to Adriamycin resulted in complete reversal of the resistance exhibited by the MCF7 Adr cell line [7] using 50 μg/ml lonidamine. We used continuous exposure and chose a much lower concentration of 1 μg/ml, well below the concentration at which lonidamine itself is cytotoxic. It is probable that higher concentrations of lonidamine would be achievable and might reverse resistance to a greater extent. MGH-U1R may also display other mechanisms of drug resistance. Possible candidates include altered topoisomerase II expression and glutathione-*S*-transferase π. Differences in topoisomerase II expression have been described in testis and bladder cancer cell lines [19] and glutathione-*S*-transferase π levels in multidrug-resistant human breast cancer cells [2].

Lonidamine has been shown in clinical studies to be non-toxic with no major side effects, and is well tolerated orally [35, 40]. Studies of lonidamine in combination with chemotherapy in tumour types such as non-small cell lung cancer [4] and malignant glioma did not result in any potentiation of systemic side effects. Lonidamine in combination with Adriamycin has been used in the adjuvant treatment of recurrent papillary carcinoma of the urinary bladder [20]. In this report 25 patients were included in a double-blind study, in which the combination of oral lonidamine plus Adriamycin was compared with Adriamycin alone. The combination of intravesical lonidamine with Adriamycin or epirubicin has not been attempted in clinical trials in bladder cancer. Lonidamine is dissolved in dimethylsulphoxide, an agent instilled intravesically to treat

interstitial cystitis [50]. This study indicates that a combination of lonidamine with an anthracycline might be of benefit in patients with resistant superficial bladder cancer.

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