Effect of calcium antagonists on the chemosensitivity of two multidrug-resistant human tumour cell lines which do not overexpress P-glycoprotein

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Summary We have examined the ability of eight compounds to enhance Adriamycin (ADM) sensitivity of two human tumour cell lines (a small cell lung cancer cell line, NCI-H69, and a fibrosarcoma cell line, HT1080) and their multidrug-resistant variants. The resistant cell lines (H69AR and HT1080/DR4) do not overexpress P-glycoprotein. Verapamil, nicardipine, perhexiline maleate, chloroquine, tamoxifen, clomiphene, prenylamine and trifluoperazine were tested alone and in combination with ADM for their cytotoxic effects. No major differences in sensitivity between the parent and resistant cell lines were noted when these agents were tested alone, except for HT1080/DR4 cells which exhibited a slight collateral sensitivity to nicardipine and H69AR cells which showed cross-resistance to chloroquine and clomiphene. When the chemosensitisers were combined with ADM no enhanced cytotoxicity of either parent cell line was observed. In HT1080/DR4 cells, verapamil showed only a modest dose-dependent chemosensitising effect while the other compounds had no effect. Verapamil and nicardipine enhanced ADM cytotoxicity in H69AR cells slightly but these effects were not dose-dependent. These results demonstrate that the reversal of drug resistance by verapamil and other calcium antagonists in a dose-dependent fashion is not an invariable property of multidrug-resistant tumour cells.

One of the major reasons for eventual treatment failure in initial responsive tumours is the appearance and proliferation of multidrug-resistant (MDR) tumour cells. A large number of mammalian cell lines have been described which exhibit the MDR phenotype and thus provide a model for the study of this clinical problem. Even though selected with a single agent, these cell lines are resistant to a wide range of chemically and functionally unrelated drugs. A variety of biochemical changes in MDR cells have been described but the most consistent alteration has been the increased expression of a high molecular weight plasma membrane glycoprotein (Gerlach et al., 1986). This protein was first described by Ling & Thompson (1974) in Chinese hamster ovary (CHO) cells and has been termed P-glycoprotein. Considerable evidence suggests that this protein is responsible for increased efflux of drug from MDR cells (Gros et al., 1986). Enhanced P-glycoprotein expression has been detected in tumour samples from MDR patients with ovarian carcinoma (Bell et al., 1985), leukaemias (Ma et al., 1987) and fibrosarcomas (Gerlach et al., 1987) indicating that the phenomenon occurs in humans as well. While P-glycoprotein has clearly been shown to play an important role in MDR, it is unlikely that it is solely responsible for MDR in all tumour cells (Kaye & Meric, 1986). In fact, several human cell lines displaying an MDR phenotype have recently been described in which no evidence of enhanced P-glycoprotein expression can be found by immunoassay or RNA blotting methods (Slovak et al., 1988; Mirski et al., 1987; Marsh & Center, 1987; Trent et al., 1988).

Tsuruo et al. (1981) were the first to report that verapamil (VP), a calcium channel blocker, could reverse MDR in vitro. A variety of compounds have since been identified which enhance drug sensitivity in vitro and are often referred to as 'chemosensitisers'. Included in this group are such diverse compounds as nicardipine (Ramu et al., 1984c), trifluoperazine (Ganapathi & Grabowski, 1983), tamoxifen (Ramu et al., 1984a), quinidine (Tsuruo et al., 1984), reserpine (Inaba et al., 1981), chloroquine (Zamora & Beck, 1986), propranolol (Shiraishi et al., 1986), perhexiline maleate (Ramu et al., 1984a), synthetic isoprenoids (Yamaguchi et al., 1986), cyclosporin A (Slater et al., 1986) and local anaesthetics (Chlebowski et al., 1982). While most, if not all, of these compounds affect calcium metabolism, it is generally agreed that the ability of these agents to enhance chemosensitivity results from interactions with a membrane transport system and not via modulation of calcium fluxes (Ramu et al., 1984a, c; Kessel & Wiëberding, 1984). Despite the fact that the mode of action of chemosensitisers is not yet understood (Hindenburg et al., 1987; Harker et al., 1986; Ganapathi et al., 1986), these chemicals are being evaluated in clinical trials (Ozols et al., 1987; Dalton et al., 1987, 1989; Tormey et al., 1982).

Many of the cell lines in which reversal of resistance has been demonstrated have also been shown to express enhanced levels of P-glycoprotein (Zamora & Beck, 1986; Hamada et al., 1987; Tsuruo et al., 1981). The present study was undertaken to determine whether or not Adriamycin (ADM) resistance could be pharmacologically reversed in two MDR human tumour cell lines which lack elevated P-glycoprotein.

Materials and methods

Drugs

Chloroquine diphosphate, clomiphene citrate, nicardipine, perhexiline maleate, tamoxifen citrate, trifluoperazine dihydrochloride, prenylamine, verapamil hydrochloride and MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide] were obtained from Sigma Chemical Co. (St Louis, MO, USA). Adriamycin (Doxorubicin, HCl; ADM) was obtained from Adria Laboratories (Burlington, Ontario) through the Kingston Cancer Clinic pharmacy.

Cell culture

The human small cell lung cancer (SCLC) cell line NCI-H69 (H69) was kindly provided by Dr J. Minna (National Cancer Institute, Bethesda, MD, USA). A MDR variant of H69, designated H69AR, was established by culturing the sensitive cells in increasing concentrations of ADM and has been described previously (Mirski et al., 1987). These cell lines were maintained in RPMI 1640 medium (GBCO) supple-
mented with 5% heat-inactivated fetal bovine serum (FBS) and 4 mM l-glutamine. H69AR was challenged biweekly with 0.6 μM ADM.

The human fibrosarcoma cell line HT1080 and the derivation of its MDR variant HT1080/DR4 are described elsewhere (Slovak et al., 1988). These cell lines were maintained in minimal essential medium (GIBCO) supplemented with 10% FBS, 4 mM l-glutamine and non-essential amino acids (GIBCO) (0.1 mM). HT1080/DR4 cells were cultured in 0.2 μM ADM at all times except for the 48 h before drug additions when the medium was replaced with that containing no ADM. All cell lines were cultured at 37°C under 5% CO2. Cultures were checked for Mycoplasma contamination using the 4',6-diamidino-2-phenylindole DNA-binding method (Russell et al., 1975) and found to be negative.

**MTT assay**

Chemosensitivity was determined using the MTT assay (Mosmann, 1983; Cole, 1986; Mirski et al., 1987). The human fibrosarcoma cell lines were harvested by incubation in citrated phosphate-buffered saline (PBS) for 10 min at 37°C, while H69 and H69AR cells were harvested by centrifugation. After resuspension in fresh medium, the cells were plated in a volume of 100 μl at 5 x 10^5 cells per well for HT1080 and HT1080/DR4 and 2.5 x 10^6 cells per well for H69 and H69AR in 96-well plates. These cell densities were chosen because they allowed exponential growth throughout a five-day assay period. The plates were incubated at 37°C overnight and then ADM and chemosensitisers were added in a total volume of 100 μl and the plate incubated at 37°C for a further five days.

The chemosensitisers were dissolved in methanol, except for trifluoperazine which was dissolved in dimethyl sulphoxide and chloroquine which was dissolved in tissue culture medium. Stock solutions were diluted in RPMI 1640 5% FBS medium as required and were prepared freshly for each experiment. Three hours before the end of the drug exposure time, 100 μl of medium was removed from each well, 25 μl of MTT (2 mg/ml) in PBS) were added and the plate incubated at 37°C for an additional 3 hours. One hundred μl of 1 N HCl:isopropanol (1:24) was added to each well followed by thorough mixing with a multichannel pipette and a further 1 h incubation at 37°C to aid solubilisation of the formazan crystals. The absorbance at 570 nm was determined using a Dynatech MR600 microplate reader. Within each experiment, determinations were done in quadruplicate and each drug was tested in two or more separate experiments. Controls were done with cells plus vehicle (baseline). In the combination experiments, wells with cells plus chemosensitisers but no ADM were also included as controls.

Results are expressed as a percentage of the baseline absorbance at 570 nm and an ID50 was defined as the dose of drug which reduced absorbance to 50% of control values. Only those combination experiments in which the chemosensitiser alone reduced the baseline absorbance by less than 20% are reported. An ADM dose-response curve was determined in each experiment. Throughout these series of experiments the relative resistance of H69AR cells to ADM ranged from 20 to 141-fold while the relative resistance of HT1080/DR4 cells ranged from 45 to 333-fold. The mean relative resistance (± s.e.m.) of H69AR and HT1080/DR4 cells was 66 ± 12 (n = 12) and 88 ± 21 (n = 14), respectively. In some experiments, low doses of ADM appeared to cause a small (20%) stimulatory effect on the growth of H69AR cells. This effect was not seen with the other cell lines. The basis of this stimulation is unknown but may be similar to that described by Grace et al. (1987).

**Discussion**

The development of drug resistance in cancer patients is widely recognised as a major impediment to successful chemotherapy. Thus the discovery that the calcium channel blocker VP could reverse MDR in an experimental system was an important finding since it has provided a strategy for treating this clinical problem.

Many structurally diverse chemosensitisers in addition to VP have been identified in experimental systems. However, no single agent has been demonstrated to be clearly superior to others nor has the mode of action of these compounds been elucidated. The current sequence suggests that chemosensitising agents may act by different mechanisms (Kessel, 1986). Despite this lack of information, VP has made its way into several clinical trials. Ozols et al. (1987) were unable to find any chemosensitising effect of VP for ADM in eight drug resistant ovarian cancer patients. On the other hand, Dalton et al. (1987, 1989) have demonstrated a therapeutic benefit of VP for P-glycoprotein-positive B-cell neoplasms. These findings demonstrate that it is not yet possible to predict the clinical usefulness of these agents in a given group of patients. Furthermore, there is insufficient information to allow the
Figure 1: Effect of chemosensitisers on the growth of human fibrosarcoma cell lines, HT1080 (○) and multidrug resistant HT1080/DR4 (●) as measured by the MTT assay. Cells were set up at 5 × 10³ cells per well on day 0, drugs added on day 1 and cell viability measured on day 5. Within each experiment, assays were done in quadruplicate. Each point represents the mean of results from two or more individual experiments which varied < 10%. Error bars represent the s.d. where three or more experiments were performed.

Figure 2: Effect of chemosensitisers on the growth of small cell lung cancer cell lines, H69 (○) and multidrug resistant H69AR (●) as measured by the MTT assay. Cells were set up at 2.5 × 10⁴ cells per well on day 0, drugs added on day 1 and cell viability measured on day 5. Within each experiment, assays were done in quadruplicate. Each point represents the mean of results from two or more individual experiments which varied < 10%. Error bars represent the s.d. where three or more experiments were performed.
rational selection of the optimal chemosensitiser for each group.

There are many possible explanations for the contrasting results reported by Ozols et al. (1987) and Dalton et al. (1987), including methodological differences. It is also possible that only certain tumour types respond to the effects of chemosensitising agents. Another possibility is that only drug resistance associated with P-glycoprotein is susceptible to reversal by VP and other compounds (Croop et al., 1987). The results of the present study support this latter idea since, of eight different agents tested, only VP (in the case of HT1080/DR4 cells) and VP and nicardipine (in the case of H69AR cells) had any ability to enhance ADM cytotoxicity. In all instances, the enhancing effect was modest and, in the H69AR cells, the effects were not dose-dependent, suggesting a limited potential for calcium antagonists to reverse resistance in these cell lines. Neither the HT1080/DR4 cell line nor the H69AR cell line overexpress P-glycoprotein (Trent et al., 1988). Further support for this idea comes from the study of Beck et al. (1987) who found that VP did not reverse resistance in an ‘atypical’ (non-P-glycoprotein-containing) MDR human leukaemia cell line. The results of individual experiments are given where there are less than three experiments were done. Within each experiment, assays were done in quadruplicate.

### Table I Effect of chemosensitisers on ADM sensitivity of human fibrosarcoma cell lines HT1080 and HT1080/DR4

| Drug            | Concentration (μM) | HT1080  | HT1080/DR4 |
|-----------------|--------------------|---------|------------|
| Verapamil       | 10                 | 7.43 (± 0.57) | 2.63 (± 1.42a) |
|                 | 3                  | 4.21 (4.45, 3.96) | 2.48 (1.75, 3.20) |
|                 | 1                  | 1.42 (1.11, 1.80) | 1.46 (1.11, 1.80) |
| Chloroquine     | 10                 | 1.27 (± 0.15) | 1.12 (± 0.08) |
|                 | 3                  | 1.11 (0.80, 1.41) | 1.01 (± 0.51) |
|                 | 1                  | 1.23 (± 0.73) | 1.09 (± 0.86) |
| Trifluoperazine | 3                  | 1.04 (1.27, 0.80) | 1.20 (1.26, 1.13) |
|                 | 1                  | 1.21 (1.42, 1.00) | 1.01 (1.13, 0.89) |
| Clomiphene      | 3                  | 1.06 (± 0.30) | 1.41 (± 0.15) |
|                 | 1                  | 1.05 (± 0.14) | 1.13 (± 0.14) |
| Tamoxifen       | 1                  | 0.91 (1.00, 0.80) | 0.91 (0.99, 0.96) |
|                 | 0.3                | 0.88 (0.63, 1.12) | 1.01 (1.11, 0.90) |
| Prenylamine     | 1                  | 1.06 (1.00, 1.12) | 1.06 (1.00, 1.12) |
|                 | 0.3                | 1.00 (1.00, 1.00) | 1.00 (1.00, 1.00) |

*Dose modifying factor is defined as the ID50 ADM: ID50 (ADM + chemosensitiser); a.s.d. given in parentheses where three or more experiments were performed. The results of individual experiments are given where there are less than three experiments were done. Within each experiment, assays were done in quadruplicate.

### Table II Effect of chemosensitisers on ADM sensitivity of small cell lung cancer cell lines H69 and H69AR

| Drug            | Concentration (μM) | HT69  | H69AR  |
|-----------------|--------------------|-------|-------|
| Verapamil       | 10                 | 4.86 (± 1.96) | 0.91 (0.82, 1.00) |
|                 | 3                  | 1.81 (± 1.02) | 1.08 (± 0.52) |
|                 | 1                  | 2.42 (± 0.79) | 1.24 (± 0.70) |
| Chloroquine     | 3                  | 1.34 (1.41, 1.26) | 1.84 (2.54, 1.13) |
|                 | 0.3                | 2.81 (± 2.48) | 0.94 (± 0.34) |
| Trifluoperazine | 1                  | 1.63 (1.25, 2.00) | 2.32 (2.86, 1.78) |
|                 | 0.3                | 1.27 (± 0.59) | 0.80 (± 0.41) |
| Clomiphene      | 1                  | 1.74 (± 0.70) | 1.69 (1.80, 1.58) |
|                 | 0.3                | 0.99 (± 0.59) | 0.85 (± 0.20) |
| Tamoxifen       | 3                  | 1.23 (± 0.35) | 1.13 |
|                 | 1                  | 2.30 (1.42, 3.17) | 1.49 (± 1.13) |
| Prenylamine     | 1                  | 2.59 (± 0.72) | 1.63 (± 0.52) |
|                 | 0.3                | 3.33 (± 1.64) | 1.85 (± 1.50) |
| Nicardipine     | 3                  | 4.59 (5.61, 3.56) | 1.09 (± 0.18) |
|                 | 1                  | 4.22 (3.98, 4.48) | 1.41 (1.42, 1.40) |
| Perhexiline     | 1                  | 2.44 (± 1.02) | 0.94 (± 0.58) |
|                 | 0.3                | 3.93 (5.04, 2.81) | 1.32 (0.63, 2.00) |

*Dose modifying factor is defined as the ID50 ADM: ID50 (ADM + chemosensitiser); a.s.d. given in parentheses where three or more experiments were performed. The results of individual experiments are given where there are less than three experiments were done. Within each experiment, assays were done in quadruplicate.
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