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New chemotherapeutics in malignant mesothelioma: effects on cell growth and IL-6 production

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Abstract *Purpose*: The benefits of chemotherapy can be assessed in terms of tumour shrinkage, prolongation of life or simply palliation of symptoms. In the study reported here, in vitro correlates of these parameters were sought as a rational guide to the choice of newer agents in the clinic. *Methods*: The cytotoxicity and effects on IL-6 production of ten chemotherapy agents representing four different classes of drugs were tested against a panel of five mesothelioma cell lines. *Results*: The mesothelioma cells were more sensitive to the action of irinotecan (and its active metabolite SN38) and gemcitabine than the control cell lines. Gemcitabine and to a lesser extent irinotecan inhibited the secretion of the proinflammatory cytokine IL-6 at concentrations of each drug that produced only small decreases in cell viability. This effect was not seen in cells treated with docetaxel or vindesine. Higher doses of gemcitabine and irinotecan caused a surge in IL-6 release and this was not due to release of intracellular stores of IL-6 through lysis of the cells. *Conclusions*: These results suggest that irinotecan and gemcitabine are not only more likely to be active against mesothelioma than other new chemotherapy agents but may also produce a palliative effect in nonresponders to these agents by decreasing the secretion of IL-6.

Key words Malignant mesothelioma · Chemotherapy · Gemcitabine · Irinotecan · Interleukin-6

Abbreviations *ABTS* 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) · *ELISA* enzyme linked immunosorbent assay · *IL-6* interleukin 6 · *MM* malignant mesothelioma · *MTT* 3-(4,5-dimethylthiazo-2-yl)-2,5-diphenyltetrazolium bromide · *PBS* phosphate-buffered saline · *SN38* 7-ethyl-10-hydroxycamptothecin

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Introduction

Malignant mesothelioma (MM) is an incurable tumour of serosal surfaces that generally occurs following exposure to asbestos. It is an uncommon tumour but the incidence of the disease is rising and is not expected to peak until 2010 [18]. This continuing increase, despite strict controls on the use of asbestos in the Western world since the 1960s, is related to the long latency period between exposure and disease occurrence. The increasing incidence of MM has not been accompanied by any significant advances in the management of this lethal disease and the average survival from the time of diagnosis remains around 9 months [19]. MM is an aggressive tumour usually associated with major systemic features such as weight loss and fever. Surgical treatment of MM is palliative and limited to those few patients in whom the disease is diagnosed early. MM is also generally regarded as resistant to radiotherapy and though immunological strategies for treatment have shown some promise they have not yet made any significant impact on survival [24]. Current approaches to the management of MM therefore rely upon chemotherapy.

A recent review of the use of chemotherapy in MM reported that standard drugs result in response rates that are usually less than 20% and there is little evidence of any increased response using combination chemotherapy [21]. A large number of new chemotherapy agents have been developed in the last decade. Many of these have shown activity against a wide range of solid tumours but have not been tried in MM. Although it would be desirable to test each of these drugs alone and in combination in MM, this is difficult given the small numbers of patients who are eligible for such trials in any one center. In order to examine the effects of these agents, we tested the activity of the more commonly used new drugs against a panel of established MM cell lines.

The benefits of chemotherapy should not be exclusively assessed by measuring response rates as determined by tumour shrinkage. Where a cancer is not curable, the aim of treatment can be prolongation of life or palliation of symptoms. Quality of life measures are increasingly becoming a routine component of the assessment of the effectiveness of new treatments. In two studies of the agents currently in clinical use, one of gemcitabine alone [2] and one of gemcitabine in combination with cisplatin [6], symptomatic improvement in a proportion of both responders and nonresponders was found. MM patients often exhibit severe systemic features and it is thought that one of the major mediators is the cytokine, IL-6. IL-6 is found in the pleural fluid of patients [16] and in the supernatants of both human [22] and murine MM cell lines [1]. It is a proinflammatory cytokine and its blockade with anti-IL-6 antibodies has been shown to reduce cachexia and improve clinical status in mice with MM [1].

In the current study, we therefore addressed two issues. Firstly, whether new chemotherapy agents with different mechanisms of action were effective against MM cells. Secondly, whether these agents altered the production of IL-6.

Materials and methods

Cell lines

A panel of five human MM cell lines, established in our laboratory from the pleural fluid of patients with the disease, were used. These cell lines, designated Ju77, Lo68, No36, One58 and Sty51 have been previously described [12]. None of the patients from whom the cell lines were derived had been exposed to chemotherapeutic agents. Two cell lines were purchased from ATCC (Rockville, Md.) for use as controls: HT29, a human colon adenocarcinoma (ATCC HTB38), and A549, a human lung adenocarcinoma (ATCC CCL185). These cell lines are representative of tumours that are generally drug-resistant but have shown some responses to newer chemotherapy agents. They have previously been shown to have a similar degree of drug resistance to MM cell lines in vitro [4].

Cells were maintained in RPMI-1640 containing 5% fetal calf serum (Life Technologies, Melbourne, Australia), 5×10^{-5} *M* 2-mercaptoethanol, 20 m*M* HEPES buffer, 100 IU/ml benzylpenicillin (CSL, Perth, Australia) and 50 μg/ml gentamicin (David Bull Laboratories, Melbourne, Australia), in water-saturated air containing 5% CO_2 at 37 °C. This growth medium is referred to here as RF-5. All cells were checked at 3-monthly intervals for mycoplasma and remained negative to testing.

Chemotherapy agents

Vincristine (Delta West, Perth, Australia) and vinblastine (David Bull Laboratories) were purchased. Paclitaxel (Bristol-Myers Squibb, Princeton, N.J.), docetaxel (Rhone-Poulenc Rorer, NSW, Australia), vindesine and gemcitabine (Eli Lilly Australia, NSW), vinorelbine (Pierre Fabre Oncology, Winchester, UK), irinotecan and SN38 (Pharmacia and Upjohn, Kalamazoo, Mich.), and topotecan (SmithKline Beecham Pharmaceuticals, Victoria, Australia) were all donated by their respective manufacturers. All drugs were stored as advised by the manufacturers, made up if necessary in recommended solvents as stock solutions and used within the period of stability for each agent as determined by the company. Dilutions of each drug were made fresh on the day of use in RF-5 from these stocks.

MTT assay

Drug sensitivities for each cell line were determined by use of the MTT (Sigma-Aldrich, NSW, Australia) assay modified from the method described by Mossman [17]. Cells were seeded in 100 μl RF-5 at appropriate concentrations to maintain logarithmic growth for the duration of the assay in 96-well flat bottomed tissue culture plates. The plates were incubated for 24 h to ensure stable growth and then dilutions of drug were added to each well in triplicate to make a total volume of 200 μl. Control wells were made for each plate with no drug or with no cells. Plates were then incubated for 72 h after which 50 μl MTT (2 mg/ml) was added followed by a further 4-h incubation. Plates were then centrifuged at 2000 rpm for 5 min after which the medium was carefully aspirated from each well. Dimethyl sulphoxide (100 μl) was added to solubilize the formazan crystals and each plate placed on a shaker for 30 min. Optical densities were determined using a SpectraMax 250 plate reader (Molecular Devices) at 570 nm. Plates were blanked on the cell-free medium containing wells that had otherwise been treated in an identical manner. All experiments were repeated at least three times. The standard deviation for triplicate values for each experiment was always less than 10%. The IC_{50} for each cell line treated with a particular drug was calculated as being the concentration of drug that resulted in a 50% reduction in the optical density of treated cells compared to untreated cells.

IL-6 ELISA

The IL-6 assay was performed using the Pelikine Compact human IL-6 ELISA kit (CLB, Amsterdam, Netherlands) according to the manufacturer's protocol. Briefly, 96-well plates were incubated at room temperature overnight with 100 μl/well of monoclonal anti-human IL-6 antibody in coating buffer (0.1 *M* $Na_2CO_3/NaHCO_3$, pH 9.6). The plate was washed five times with PBS and then 200 μl blocking buffer was added to each well which was followed by incubation for 1 h at room temperature. The plate was then washed five times in PBS with 0.005% Tween 20 (Sigma-Aldrich, NSW, Australia). A 100-μl aliquot of each sample to be assayed was added to each well and also IL-6 standard solutions ranging from 0.6–450 pg/ml were used to generate a standard curve. After a 1-h incubation, the plate was washed as above and 100 μl biotinylated IL-6 antibody solution added for a further hour. Following further washes, 100 μl streptavidin-horseradish peroxidase conjugate solution was added for 30 min and the plates washed. ABTS solution (100 μl) was added and following incubation for 30 min at 37 °C, the plate was read at 405 nm on a SpectraMax 250 plate reader (Molecular Devices) and the concentration of IL-6 for each sample calculated from the standard curve.

Samples for use in the ELISA were prepared by seeding of cells in 96-well plates with or without chemotherapy as described previously. As in the previous experiments chemotherapy was added 24 h after cell seeding. For cell lysis experiments 200 μl distilled water was added to each well and lysis of cells was confirmed by visual inspection. The medium from these assays was removed at the appropriate time and stored at −20 °C until the ELISA was performed. The concentration of IL-6 given for each cell line represents the mean value generated from three separate experiments. The results from the effects of chemotherapy on IL-6 production are representative of two or more experiments.

Results

In vitro drug sensitivity in MM

The concentration of drug required to reduce cell viability by 50% (IC_{50}) for four classes of drug was calculated using the MTT assay. The five MM cell lines had IC_{50} values for gemcitabine ranging from 4.4×10^{-9} to

2.0×10^{-8} *M*, a greater than fourfold difference in sensitivity between the most- and least-sensitive cell line (Fig. 1A). The IC_{50} value for the lung cancer cell line, A549, fell within this range (8.7×10^{-9} *M*). By contrast, the colon cancer cell line, HT29, had an IC_{50} value more than seven times higher than any other examined cell line (1.5×10^{-7} *M*).

Each of the five MM cell lines was more sensitive to the action of irinotecan than the two control cell lines (Fig. 1B). The IC_{50} value for the MM cell lines differed by less than threefold (3.7×10^{-6} to 9.2×10^{-6} *M*), whereas the control cell lines fell outside this range (2.4×10^{-5} *M* for A549 and 1.7×10^{-5} *M* for HT29) and this difference was statistically significant ($P < 0.005$; Mann-Whitney *U*-test). The active metabolite of irinotecan, SN38, produced a similar pattern of activity with the MM cell lines being more sensitive than the control cell lines. The IC_{50} value for SN38 was approximately 1000 times lower than that of irinotecan for all cell lines examined.

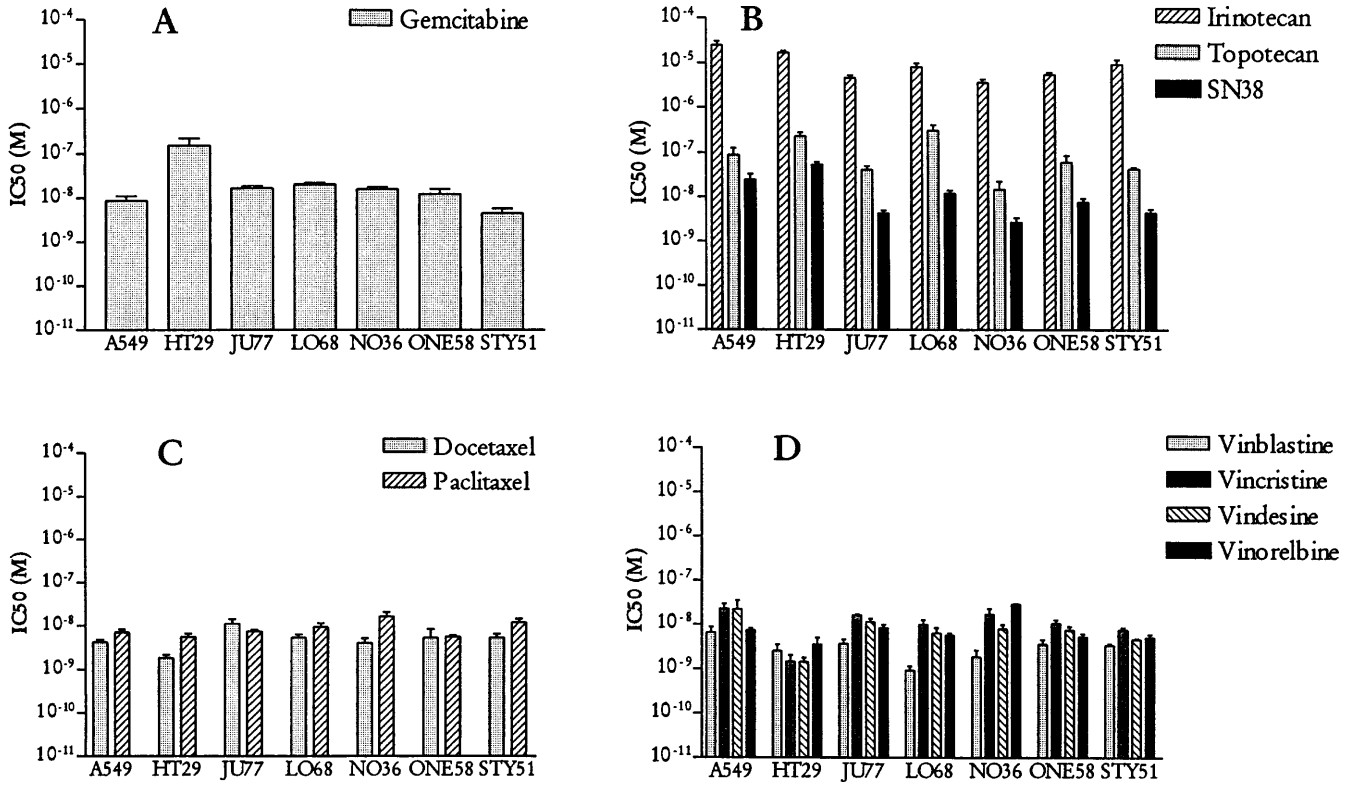
For topotecan the range of IC_{50} values for the MM cell lines was 1.5×10^{-8} to 3.1×10^{-7} *M*, a 20-fold difference in sensitivity (Fig. 1B). The two control cell lines were more sensitive to topotecan than one MM cell line (8.9×10^{-8} *M* for A549 and 2.3×10^{-7} *M* for HT29) but less sensitive than the other four MM cell lines.

The range of sensitivities of the cell lines to paclitaxel was 5.6×10^{-9} to 1.7×10^{-8} *M* (Fig. 1C). Both of the control cell lines were more sensitive to this agent than all but one of the MM cell lines. For docetaxel, HT29 was the most sensitive (1.8×10^{-9} *M*) and the five MM cell lines had IC_{50} values ranging from 4.1×10^{-9} to 1.1×10^{-8} *M*. The IC_{50} value for A549, fell within this range at 4.3×10^{-9} *M*.

The four vinca alkaloid drugs examined generally produced a consistent pattern of response against all seven cell lines (Fig. 1D). HT29 was the most sensitive to three of the four agents, the exception being vinblastine. Similarly, STY51 was the most sensitive MM cell line to the same three vinca alkaloids. In contrast, A549 and the MM cell lines JU77 and NO36 were the least sensitive to these drugs overall. NO36 was strikingly less sensitive than all the other cell lines to vinorelbine, as was A549 to vindesine.

On a molar basis, all five of the MM cell lines were most sensitive to the older agent vinblastine. Vinorelbine was the next most potent vinca alkaloid by this criterion in four of the five MM cell lines. The exception was NO36, which displayed the least sensitivity to vinorelbine of all of these agents on a molar basis.

Fig. 1A–D The effect of four different classes of drug on the viability of five MM cell lines and two control cell lines were determined in vitro. Gemcitabine (**A**), topoisomerase I inhibitors (**B**), taxanes (**C**) and vinca alkaloids (**D**) were compared. Dose response curves were plotted for each experiment and the concentration of drug that reduced cell viability by 50% (IC_{50}) was calculated. Each IC_{50} value is the mean (±SE) from at least three different experiments



IL-6 secretion from MM cells

The five MM cell lines produced different amounts of IL-6 when grown in culture for 96 h (Fig. 2). The

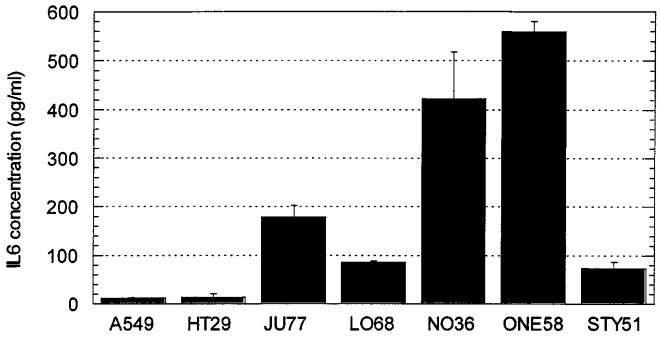


Fig. 2 IL-6 secretion from two control cell lines (A549 and HT29) and five MM cell lines. The values shown are the means (±SE) from three different experiments

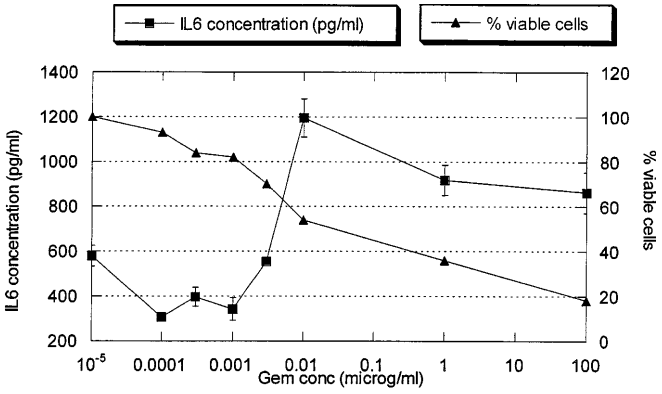


Fig. 3 IL-6 secretion was compared to cell viability in NO36 cells treated with various concentrations of gemcitabine. Relative cell growth was determined from the MTT assay by dividing the optical density of treated cells with those of untreated cells. The values shown are the means (±SE) from two experiments. For cell viability the standard deviation from the mean for all values was less than 10%

greatest producers of IL-6 were ONE58 and NO36 while STY51 produced the least. The control cell lines A549 and HT29 produced minimal amounts of IL-6. The concentration of IL-6 for each cell line was determined from the final concentration present in the growth medium following 96 h of incubation. It should be noted that all cells were seeded at the same density but because of variations in proliferation rates between the cell lines, the final cell numbers would have varied following 96 h of growth. The doubling times for the five MM cell lines range from 25.3 to 33 h. The cell lines ONE58 and NO36 were two of the slower growing cell lines and the difference in production of IL-6 between these cell lines and the others would have been greater if total cell numbers had been taken into account. The MTT assay was carried out on the cells used in this experiment and provided an indirect measurement of cell numbers. At the completion of this experiment the number of viable ONE58 cells was two-thirds that of the fastest growing cell line, JU77, and the number of NO36 cells was three-quarters that of JU77 cells.

IL-6 secretion from MM cells treated with chemotherapy

Treatment of ONE58 and NO36 cells with concentrations of gemcitabine that produced only a small decrease in cell viability resulted in a decrease in the IL-6 concentration by up to one-half that released by untreated cells (Fig. 3). This effect was present when low concentrations of the drug sufficient to produce a 5–30% reduction in relative cell growth were used. With increasing doses of gemcitabine, there was an increase in the concentration of IL-6 released by the cells. The IL-6 levels were higher than in untreated cells even when the chemotherapy dose was sufficient to produce an 80% decrease in viable cells. Gemcitabine caused this pattern of IL-6 secretion in all five MM cell lines (data not shown).

The effect of the timing of the addition of chemotherapy was examined to ascertain if this effect was related to whether cells were in active growth phase and producing IL-6. Gemcitabine was added 24 h after or concurrently with the seeding of the MM cells (Fig. 4). A reduction in IL-6 secretion was found with low doses of chemotherapy added 24 h after cell seeding and a marked increase in IL-6 secretion with higher (and more toxic) doses of gemcitabine as was previously shown. Gemcitabine added at the time of cell seeding resulted in a similar (but generally diminished) pattern of IL-6 production. Whether cells were actively growing or not at the time of gemcitabine addition did not affect the pattern of IL-6 secretion.

To determine if the increase in IL-6 secretion was related to the release of stored IL-6 from lysed cells, the

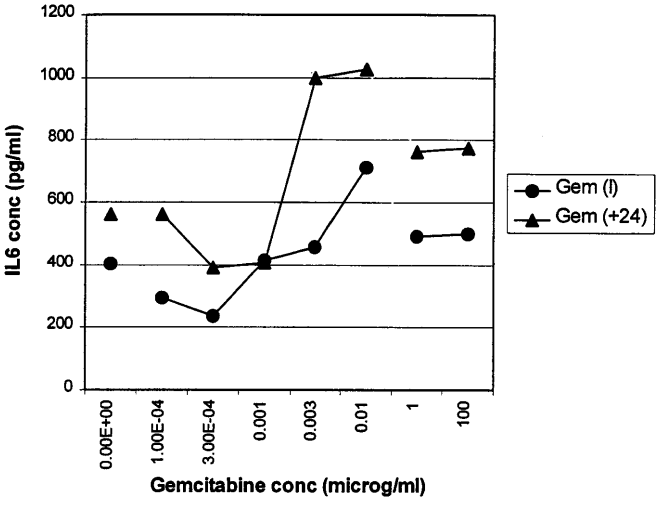


Fig. 4 IL-6 secretion by ONE58 cells treated with gemcitabine at the time of cell seeding (*Gem I*) was compared with that following addition of the drug 24 h after seeding (*Gem + 24*). The incubation time with chemotherapy was 72 h for each assay, so the total incubation time for Gem I was 72 h and for *Gem + 24* was 96 h. The IL-6 concentration therefore differs when no chemotherapy was added to the cells. Note the discontinuous *x*-axis

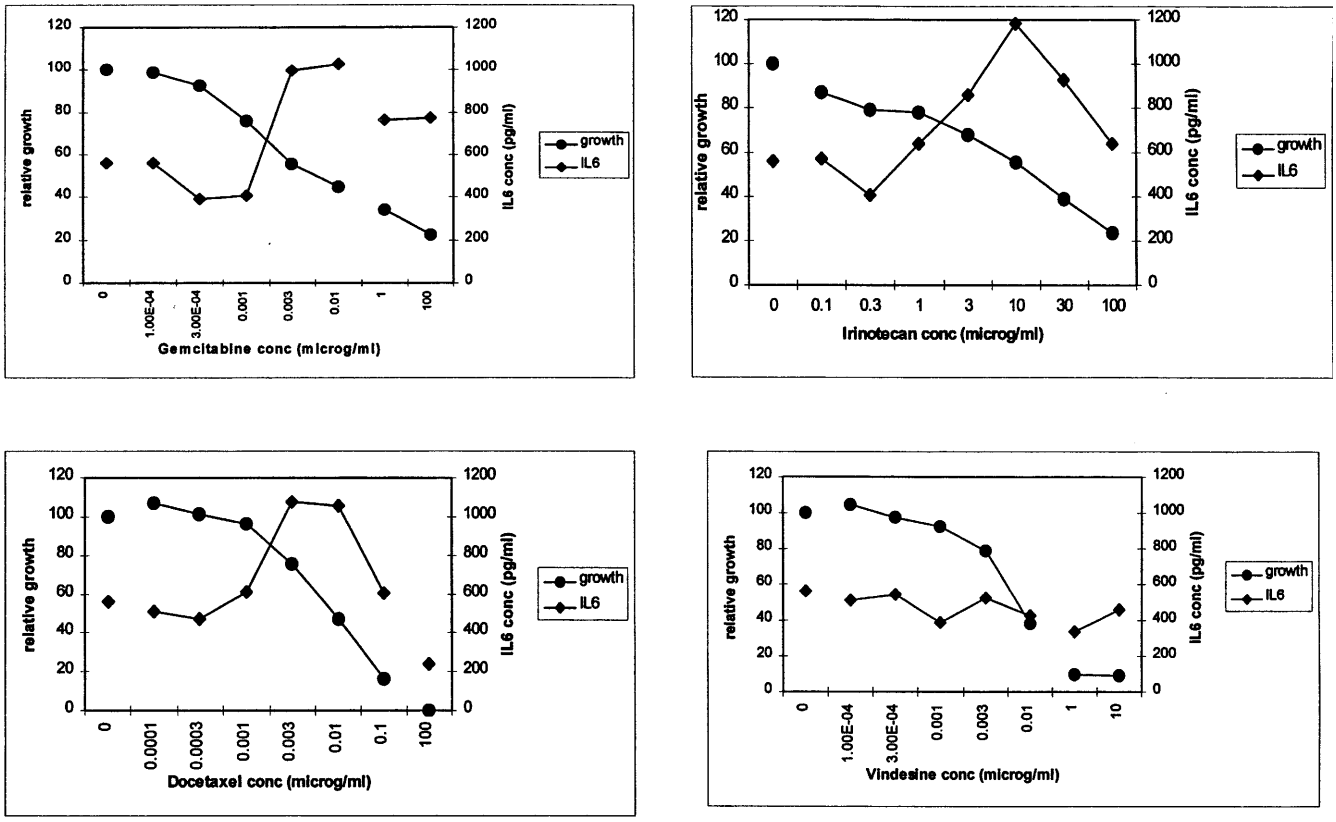


Fig. 5 IL-6 secretion by ONE58 cells was compared with total IL-6 in supernatants collected following cell lysis by hypotonic shock (*ONE58 + W*) at various time-points

medium from untreated ONE58 cells was removed at various time-points and compared with the medium removed from these cells following immediate cell lysis with water. No difference in IL-6 concentration was seen between the medium from lysed cells and that from viable cells. The concentration of IL-6 produced after 24 h of ONE58 growth was 380 pg/ml.

Chemotherapy agents from four different classes (taxanes, topo I inhibitors, vinca alkaloids and gemcitabine) were compared to determine whether the surge in secretion of IL-6 was a general effect of chemotherapy (Fig. 5). Increasing concentrations of irinotecan resulted in a similar pattern of IL-6 secretion to gemcitabine: an initial decrease in IL-6 concentration followed by a marked surge. Docetaxel caused a surge in IL-6 secretion at higher doses without the initial fall in cytokine levels, while vindesine produced a gradual fall in IL-6 levels without any surge at more toxic doses. The decrease in IL-6 concentration associated with increasing concentrations of vindesine correlated with the decrease in the number of viable cells.

Discussion

The incidence of MM continues to increase in industrialized countries despite longstanding restrictions on the use of asbestos. The failure to make any impact on the mortality rates or life expectancy for this disease using standard chemotherapy agents means the assessment of new drugs and treatments is of critical importance. Many newer drugs have shown activity in tumours such as non-small-cell lung cancer. Such malignancies had previously been thought to be as resistant to chemotherapy as MM. Assessment of these new agents in MM is restricted, however, by the low numbers of patients who are suitable for trials at any one centre. It is, therefore, useful if new agents or combinations can be first assessed in vitro and in animal models to identify those that are most likely to be active in the clinic. Our group has previously tested many older agents using the MTT assay [4]. While such a system is not ideal, in that it carries the potential to overlook clinically active drugs, it remains a useful screening tool.

MM cells are relatively sensitive to gemcitabine and irinotecan

This study demonstrated marked variation in response to the newer chemotherapy agents. One striking finding in this study was of a significantly higher IC_{50} value for HT29 cells treated with gemcitabine as compared to all other cell lines. This value was seven times higher than in any of the MM cell lines indicating that these were relatively sensitive to gemcitabine. This finding concurs with the results of clinical studies with this agent demonstrating efficacy against MM. As a single agent, gem-

citabine has achieved response rates of 31% [2] and 11% [15] and, when combined with cisplatinum, 47.6% [6].

Irinotecan was significantly more active against the MM cell lines than against the two control cell lines. This difference was as much as six times for one MM cell line compared to A549. The other camptothecin tested, topotecan, had a more even distribution of IC_{50} values for all cell lines examined indicating similar sensitivity. Topotecan has previously been found in a clinical setting to have no significant activity against MM [11]. Irinotecan has not yet been tested as a single agent in a clinical study, although activity has previously been demonstrated against human MM colony-forming units [23].

The MM cells were also more sensitive to the active metabolite of irinotecan, SN38, than the control cell lines. SN38 was approximately 1000 times more potent than irinotecan, as has been previously reported. This agent was therefore the most active of the topo I inhibitors against MM, but its activity is dependent on adequate levels of the enzyme carboxylesterase. Our analyses of the MM cell lines has shown that carboxylesterase is not a limiting factor in determining sensitivity to this agent (unpublished observations).

MM cells are relatively insensitive to the vinca alkaloids and the taxanes

Vinorelbine, the newest of the vinca alkaloids, did not show any increased cytotoxicity compared to the three older agents. Vinblastine was the most potent of these drugs, in both relative and molar terms. All MM cell lines were less sensitive to vinca alkaloids than was HT29. In clinical trials in MM, vindesine has been found to induce response rates of 6% or less [3, 9] while neither vincristine [13] nor vinblastine [8] resulted in any response. It seems unlikely, therefore, that vinorelbine will be useful in the treatment of MM. One confounding factor in this assessment is the pharmacological profile of vinorelbine. Higher doses of vinorelbine (25–30 mg/m^2) are used to treat patients compared to vinblastine (6 mg/m^2). Vinorelbine has also been demonstrated to achieve concentrations up to 300 times higher in the lung than in plasma. These levels are 3.4 times higher than those of vindesine and 13.8 times those of vincristine [10]. Higher in vitro IC_{50} values for vinorelbine compared to other vinca alkaloids may therefore not be associated with a lower likelihood of clinical response.

In this in vitro system, the IC_{50} value for paclitaxel was similar in all seven cell lines. Docetaxel also had a narrow range of IC_{50} values with the majority of MM lines being more resistant to both taxanes than the control cell lines. Paclitaxel has been found to be inactive in two clinical studies both alone (RR = 0% and 6%) [14, 25] and in combination with cisplatin (RR = 6%) [7]. It seems likely from our results that docetaxel will not display any greater efficacy than paclitaxel against this tumour.

Chemotherapy and IL-6 production

One drug that may have activity in this disease is gemcitabine. It has been noted in two clinical studies that symptomatic improvement occurs even in some nonresponders. A palliative effect of gemcitabine has also been found in other malignancies [5]. We investigated whether this effect could be due to a decrease in the secretion of IL-6, a cytokine that may causes some of the paraneoplastic effects that accompany MM.

All of the MM cell lines secreted IL-6 and low doses of gemcitabine inhibited this by one-third to one-half. This effect was not related to the timing of the addition of gemcitabine. The level of inhibition of IL-6 production in these experiments was less than it otherwise might have been because the drug was added 24 h after the cells were seeded. If the IL-6 produced in the first 24 h is subtracted, then the effect of gemcitabine treatment is even more striking. The IL-6 produced in the 72 h of drug incubation approaches zero (10–25 pg/ml) at a dose that causes only a small decrease in cell viability.

A similar but lesser reduction in IL-6 secretion was noted for irinotecan, whereas treatment with docetaxel did not cause this at any concentration. Vindesine caused a gradual fall in IL-6 secretion that corresponded to the reduction in the number of viable cells. Neither the decrease in IL-6 production nor the surge at higher doses are therefore general features of chemotherapy.

A surge in IL-6 secretion observed at higher concentrations of some drugs was not due to the release of stored IL-6 by cell lysis. It would appear most likely that there is an increase in secretion of IL-6 by cells that have been damaged but not killed by the action of these chemotherapy agents. With docetaxel treatment the highest concentration of drug tested resulted in no viable cells at the end of the assay. In this situation, the concentration of IL-6 was similar to the amount secreted in the first 24 h before chemotherapy addition. This finding lends support to the surge being due to ''stressed'' cells rather than killed cells.

It is possible that the in vitro surge in the secretion of IL-6 would be paralleled by a clinical effect in patients treated with cytotoxic doses of these agents. Thus, higher doses may induce IL-6 release from tumour cells and exacerbate patient symptoms. Any reduction in the quality of life scores of patients undergoing treatment with these agents may therefore not be due to drug toxicity alone but to the release of IL-6. Nevertheless, in the body IL-6 is cleared rapidly so any associated symptoms may be short-lived. Furthermore, these effects may be offset in the longer term by the finding that the surge in IL-6 secretion occurs when there is increased cell death. Clinically this may translate to a reduction in tumour bulk with the longer term consequence of an improvement in wellbeing. The relationship between serum IL-6 levels, tumour load and quality of life in patients undergoing chemotherapy is the subject of ongoing studies.

In a recent clinical study of IL-6 levels in patients with MM, IL-6 levels have been found to correlate with acute phase proteins such as CRP and fibrinogen in serum [20]. Interestingly, one patient in this study with a partial response to irinotecan and cisplatin exhibited a transient fall in IL-6 levels but whether this resulted in any symptomatic improvement was not reported. This accords with our finding that irinotecan has a direct effect on IL-6 secretion.

In summary, our experiments showed that of the newer commonly available agents, irinotecan and gemcitabine have the greatest potential for the treatment of MM. Further studies to determine why MM has such a wide range of intrinsic resistance to chemotherapy are being undertaken. The finding that gemcitabine and to a lesser extent irinotecan at particular doses produce a decrease in the secretion of IL-6 when they have only a small effect on cell viability could be one explanation for the palliative effect of gemcitabine in patients who have not shown a clinical response. High doses of such agents may temporarily worsen symptoms via an IL-6 surge.

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