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Interferon-α and -γ in combination with chemotherapeutic drugs: *in vitro* sensitivity studies in four human mesothelioma cell lines

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Mesothelioma is a tumor of the serous surfaces in the thorax and abdomen. This tumor has proved to be exceptionally resistant to treatment, although a variety of multi-modality therapies have been tried. We have used four human mesothelioma cell lines, originating from diffuse asbestos-related malignant (pleural) mesothelioma, to assess *in vitro* sensitivity to five chemotherapeutic drugs, to recombinant human interferon (IFN)-α and -γ and to combined immuno-chemotherapy. The cytotoxic effects were assayed by vital dye exclusion. The drugs tested were etoposide, cisplatin, mitoxantrone, 4-epirubicin and vindesine. The combinations tested were etoposide + cisplatin, and etoposide + cisplatin + mitoxantrone. All the drugs and combinations were also tested with recombinant human (rHu) IFN-α2C (rHuIFN-α), rHuIFN-γ, and rHuIFN-α + rHuIFN-γ. The cell lines were most sensitive to mitoxantrone, 4-epirubicin and vindesine ($TC_{50} \leq 0.001$ μg/ml), and least sensitive to etoposide and cisplatin ($TC_{50} \geq 0.1$ μg/ml) used singly. There was no improvement in sensitivity when the drugs were combined. To further investigate the lack of response to cisplatin treatment, we examined the binding of cisplatin to the mesothelioma cell DNA. The tumor cell DNA bound markedly less cisplatin than human fetal fibroblast DNA. Three cell lines were tested with rHuIFN-α and rHuIFN-γ on their own or rHuIFN-α + rHuIFN-γ. They were consistently sensitive to rHuIFN-α, but the sensitivity to rHuIFN-γ varied with the cell lines. Finally, we tested two cell lines with the drugs singly and in combination, together with 0.01 μg/ml each of rHuIFN-α and rHuIFN-γ. The growth inhibitory effects of all the individual drugs and combinations were improved by the addition of rHuIFN-α and rHuIFN-γ, by as much as 30% in some cases. We conclude that mitoxantrone and 4-epirubicin each combined with IFN should be further investigated in the development of mesothelioma therapy.

Key words: Chemosensitivity, chemotherapeutic agents, DNA binding, human malignant mesothelioma, interferon, *in vitro* testing.

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Introduction

Mesothelioma is a tumor of the serous surfaces of the thorax and abdomen.[1] The development of diffuse malignant mesothelioma is almost always associated with a history of asbestos exposure[2] and is characterized by a very long latent period of 10–30 years, even 40 years in some cases. The recent increase in the number of cases matches the increased use of asbestos between the Second World War and the 1970s.

There is no standard treatment for this disease: but multimodality therapy, including debulking surgery, radiotherapy and chemotherapy, is practiced at many centres. Despite this, survival in this disease remains poor; median survival is typically less than 1 year from diagnosis.[3–5].

Various chemotherapeutic regimes have been used to treat mesothelioma patients, but without consistent success. The mean survival time has not improved for a number of years. However, diagnostic techniques have improved so that mesothelioma can now be identified at an earlier stage and be reliably distinguished from adenocarcinoma. Of the various chemotherapeutic agents tested clinically against mesothelioma, doxorubicin and cisplatin appear to be among the most effective.[3,6,7]

Interest has focused recently on the potential against cancer of the biological response modifying agents, such as interferon (IFN).[8,9] These agents can be involved directly and indirectly in the host response to malignancy.[10–12] There are numerous examples of IFN augmenting or maintaining the effects of chemotherapeutic agents, such as cisplatin,

both *in vitro* and *in vivo*.[13–15] Moreover, with the rapid development of DNA techniques, many of the biological reponse modifiers are available in recombinant form for clinical use.[11,16–18] IFN-α has proved useful as maintenance therapy for patients who have been successfully treated with radiotherapy and chemotherapy.[15,19]

We have established a number of continuously-growing human mesothelioma cell lines from fresh tumor tissue.[20] We have previously reported the sensitivity of three cell lines to recombinant human (rHu) cytokine combinations involving tumor necrosis factor, IFN-α2C and IFN-γ.[21] In this paper we report the response of our mesothelioma cell lines to five commonly-used chemotherapeutic drugs, and to rHuIFN-α and rHu-IFN-γ both individually and in combination. In particular, we investigated the effect of IFN on the sensitivity of the cells to chemotherapy.

Materials and methods

Four human mesothelioma cell lines were used in these experiments, all of which were established and characterized in our laboratory from fresh tumor tissue samples.[20] Three of the cell lines (Ml4K, M33K and M38K) were established from the primary tumors of previously untreated patients; the fourth cell line (M9K) was established from the metastatic tumor of a patient who had previously been treated with mitoxantrone and radiotherapy.

From continuously growing monolayer cultures, $1.0–1.5 \times 10^5$ cells were plated into 6-well plates (10 cm^2, Nunc) in 3 ml medium (RPMI 1640 supplemented with 10% fetal calf serum, 0.03% L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin, all from Gibco). The plates were incubated at 37°C in a humidified atmosphere with 5% CO_2 for 40–50 h, to establish exponentially growing cultures.[20] The medium was then replaced by fresh medium supplemented with the drugs and/or IFN, and incubated for a further 48–72 h before harvesting (approximately 1.5–2 doublings in the control cultures). The drugs and IFN were diluted in medium to give final concentrations of 0.0001–100 μg/ml/well. The cells were detached with 0.05% trypsin–EDTA, centrifuged (1000 r.p.m. for 5 min) and stained with Trypan Blue for 5 min. Finally the numbers of viable cells were counted.

Two series of experiments were conducted: in Series 1, the three cell lines from primary tumors were tested for 48 h with etoposide (Vepesid, Bristol Myers, UK), cisplatin (Platinol, Lääkefarmos, Finland), mitoxantrone (Novantrone, Lederle, UK), 4-epirubicin (Farmorubicin, Farmitalia, Italy) and vindesine (Eldisine, Lilly, USA), all as single agents; and in Series 2, one primary tumor cell line and the metastatic tumor cell line were tested for 72 h with etoposide, cisplatin, mitoxantrone, 4-epirubicin, rHuIFN-α (Boehringer Ingelheim, Germany; specific activity 32×10^7 U/mg) and rHuIFN-γ (Boehringer Ingelheim, Germany; specific activity 2×10^7 U/mg), both individually and in various combinations. The details of the drug doses tested and the cell lines are given in Table 1. The combinations tested were the four individual drugs with rHuIFN-α and rHuIFN-γ (0.01 μg/ml of each IFN over the range of drug concentrations); etoposide and cisplatin with and without the IFN combination; and etoposide, cisplatin and mitoxantrone with and without the IFN combination. The combined IFN dose (0.01 μg of each IFN/ml) was selected beforehand from our experiments on the sensitivity of the cell lines to the individual and combined IFNs (see Figure 2).

The results are represented as the percentage of the number of control cells that survived (percent survival) against the concentration of drug(s) used. Each point on the figures represents the mean result of at least two independent experiments of duplicate cultures. The standard deviations were 5% or less in the Series 1 experiments; and in most cases 10% or less in the Series 2 experiments.

Primary human fetal fibroblast (HFF) cultures established from fresh tissue samples were used as normal human cell controls in the cytotoxicity testing (Series 1) and in DNA binding studies. HFF cells were grown as described above for mesothelioma cell lines, but with a 20% supplement of fetal calf serum. The experiments to investigate the binding of cisplatin to DNA in the various cell lines were performed as follows: early passage HFF cells and tumor cells (1.0×10^6 cells per flask) were plated into 75 cm^2 culture flasks (Nunc), allowed to grow for 40–50 h and 0.3–15 μg/ml (1×10^{-6} to 5×10^5 M) cisplatin in fresh medium was then added for a further 48 h. The cells were harvested, washed twice with Ca^{2+}- and Mg^{2+}-free phosphate buffered saline (PBS, Gibco), and DNA isolated and treated with RNase according to standard protocols. Two separate experiments were performed, with duplicate cultures. The amount of platinum bound to the cellular DNA was measured by flameless atomic absorption spectroscopy (AAS) as described by Mustonen *et al.*[22]

Table 1. Details of cell lines, experimental design and concentrations of drugs tested in four human mesothelioma cell lines

Cell line/cell passage	Anticancer agent tested	Concentration range (μg/ml)
Series 1		
M14K[a]/p[58–74]	etoposide	0.0059–59
M33K[a]/p[9–44]	cisplatin	0.0003–30
M38K[a]/p[4–25]	mitoxantrone	0.0052–52
	4-epirubicin	0.058–58
	vindesine	0.043–85
Series 2		
M9K[b]/p[8–26]	etoposide	0.001–10
M38K[a]/p[17–49]	cisplatin	0.001–10
	mitoxantrone	0.0001–1
	4-epirubicin	0.0001–1
	rHuIFN-α	0.0001–0.1
	rHuIFN-γ	0.0001–0.1
	Combinations	
	rHuIFN-α + rHuIFN-γ	0.0001–0.1 of each
	etoposide + cisplatin	0.001–10 of each
	etoposide + cisplatin + mitoxantrone	0.001–10 of each
	rHuIFN-α + rHuIFN-γ + individual drugs	0.01 of each IFN (drug concentrations as above)
	rHuIFN-α + rHuIFN-γ + drug combinations	0.01 of each IFN (drug concentrations as above)

[a] Primary tumor cell line.
[b] Metastatic tumor cell line.

Results

The results of the first series of experiments are shown in Figure 1. The responses of the cell lines to the five drugs fell into two groups: the responses to cisplatin and etoposide in one group; and the responses to mitoxantrone, 4-epirubicin and vindesine in the other. All three cell lines were insensitive to cisplatin, the 50% toxic concentration (TC_{50}) above >0.75 μg/ml, and relatively sensitive to mitoxantrone and 4-epirubicin ($TC_{50} < 0.06$ μg/ml. The sensitivity to etoposide was similar to that of cisplatin for two cell lines but M33K appeared to be as sensitive to etoposide as to the two more effective drugs, mitoxantrone and 4-epirubicin. The sensitivity to vindesine did not show any consistent pattern. The TC_{50} values for human fetal fibroblasts, used as a normal human control cell line, were generally lower (data not shown).

The results of the second series of experiments are given in Figures 2 and 3. Figure 2 shows the effects of the various treatments on (A) cell line M38K and (B) cell line M9K. Both cell lines showed a greater degree of anti-proliferation with the combination of rHuIFN-α and rHuIFN-γ, than with either IFN alone (Figure 2). The effect of the combination was much more marked for the metastatic cell line M9K. The second panel in Figure 2 shows the sensitivity of the two cell lines to the individual drugs, additional experiments to those shown in Figure 1. In this series of experiments there was a clear difference in sensitivity to cisplatin and etoposide ($TC_{50} > 0.5$ μg/ml), on the one hand, and mitoxantrone ($TC_{50} < 0.006$ μg/ml), on the other. This pattern is the same for both cell lines; 4-epirubicin falls between the two groups. The third panel in Figure 2 shows the effect of adding 0.01 μg/ml of each of human rIFN-α and rIFN-γ to the same concentrations of drugs shown in the second panel. We chose to use IFN-α and IFN-γ in combination because our preliminary studies indicated that the cells responded at least as well to both, as to either IFN individually (data not presented).

The addition of IFN appears to improve the sensitivity of the cells to all the drugs, so that the same amount of drug will produce a greater inhibitory effect. For example, whereas 1.3 μg/ml of cisplatin inhibited the M38K cells by 50% on its own, with IFN the same amount of cisplatin inhibited the cells by 65%. The effect on M9K was

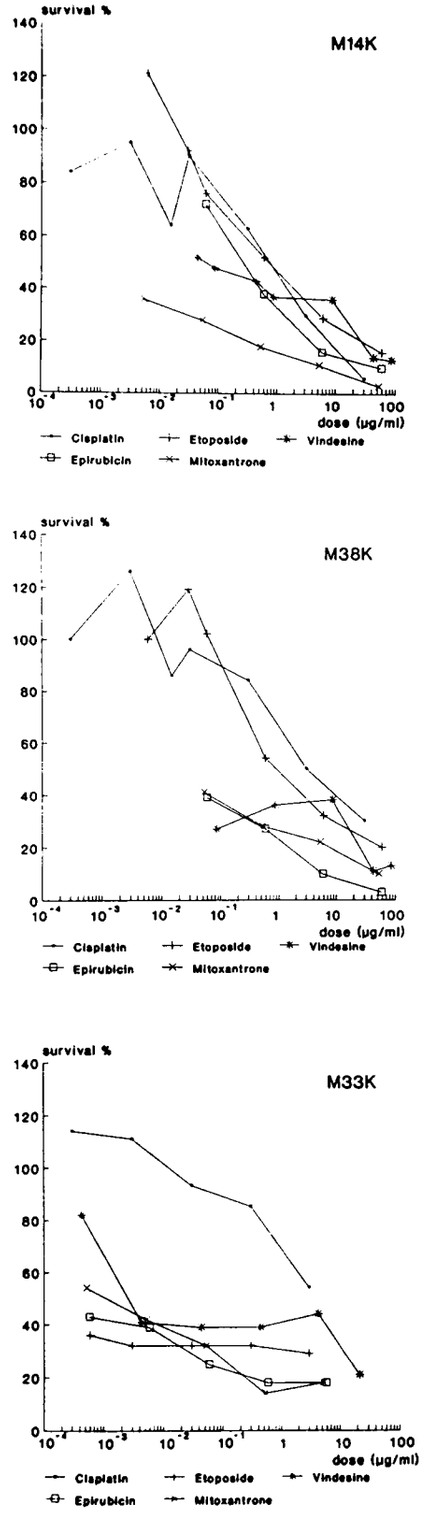

Figure 1. The effects of five chemotherapeutic drugs (etoposide, 4-epirubicin, cisplatin, mitoxantrone and vindesine) on three mesothelioma cell lines.

more pronounced: 0.004 µg/ml mitoxantrone inhibited the cells by 50% alone and by 80% when combined with IFN.

Figure 3 shows the effects of the drug combinations on the cell lines, with and without the addition of rHuIFN-α and rHuIFN-γ. No improvement in cytotoxicity over the degree achieved with the individual drugs was seen using either etoposide and cisplatin or etoposide, cisplatin and mitoxantrone. The addition of 0.01 µg/ml of rHuIFN-α and rHuIFN-γ together increased the growth inhibition effect of etoposide and cisplatin from 50 to 65% for M38K and to 75% for M9K (Figure 3A).

A very similar response was seen with the etoposide, cisplatin and mitoxantrone combination. The addition of human rIFN-α and rIFN-γ did not significantly increase the growth inhibition effect for M38K, but growth inhibition in M9K was increased from 50 to 80% (Figure 3B).

To further investigate the lack of response to cisplatin treatment, we examined the binding of cisplatin to the mesothelioma cell DNA and compared the results with those from normal human fetal fibroblasts. The amount of platinum bound to the cellular DNA in the mesothelioma cells (M14K, M33K and M38K) was clearly less than that bound to the normal fibroblast DNA (Table 2).

Discussion

The results of these sensitivity studies suggest, as already indicated in our preliminary report,[23] that mesothelioma cells appear to be more sensitive to the DNA intercalating anthracyclines, mitoxantrone and 4-epirubicin, than to either etoposide or cisplatin which have other modes of action.[9] These single agent results have clinical implications because high dose 4-epirubicin (110 mg/m^2 every 3 weeks) appears currently to be the most effective drug against mesothelioma,[7] although Rebattu and colleagues recently reported good results with high dose cisplatin (200 mg/m^2).[24] This could, however, be in accordance with the results from our experiments, where we were able to achieve the same level of cytotoxicity with cisplatin as with mitoxantrone and 4-epirubicin, but at much higher doses. Mitoxantrone, a more recent anthracenedione derivative,[25] has not proved useful in the treatment of mesothelioma,[26,27] but has only been used at the comparatively low dose of 14 mg/m^2 every 4 weeks. Treatment with 4-epirabicin is also

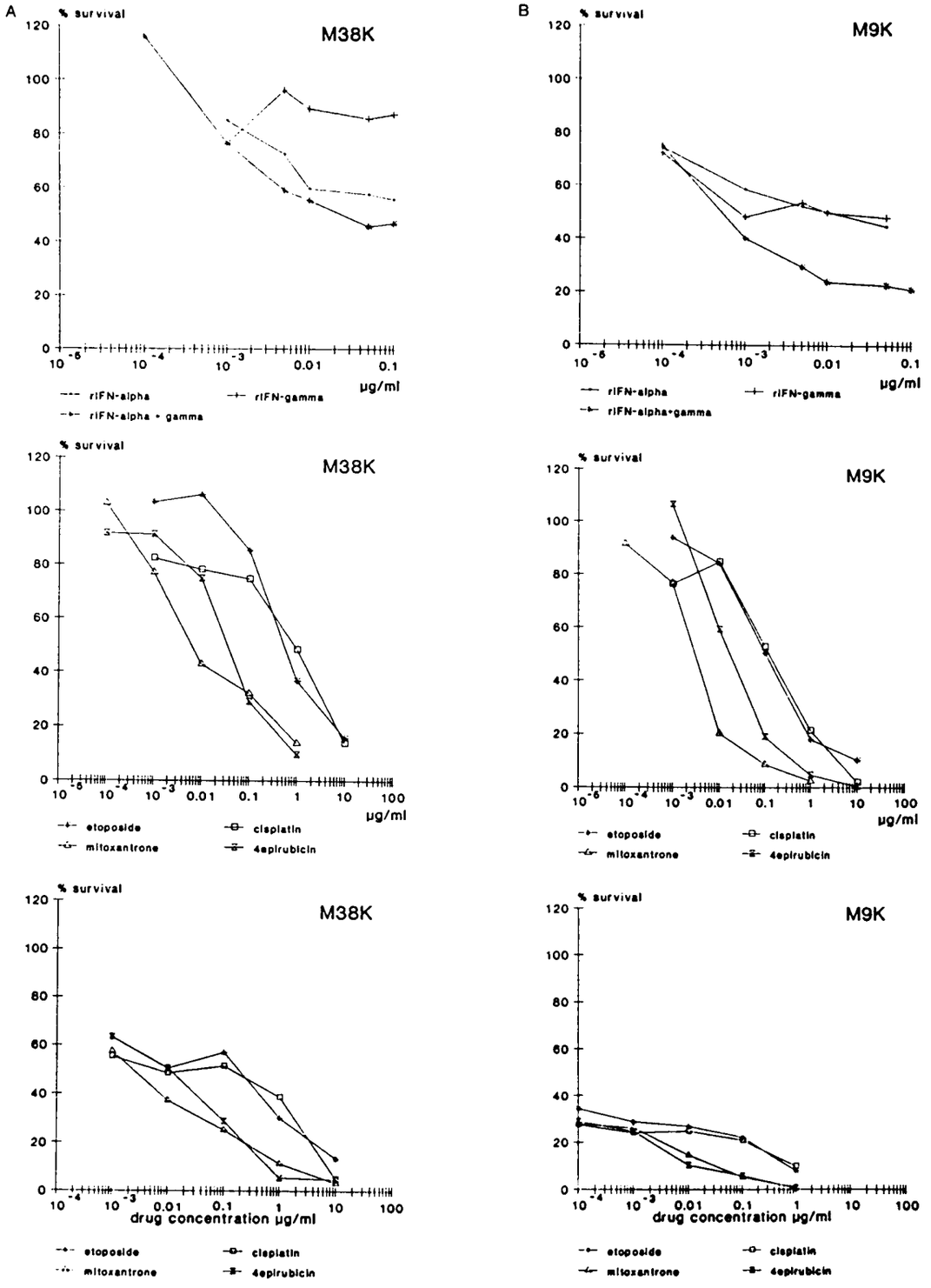

Figure 2. (A) The effects of human IFN (rHuIFN-α and rHuIFN-γ, top panel), four chemotherapeutic drugs (etoposide, 4-epirubicin, cisplatin and mitoxantrone; middle panel), and the chemotherapeutic drugs each with 0.01 µg/ml of both rHuIFN-α and rHuIFN-γ (bottom panel) on mesothelioma cell line M38K. (B) The effects of human IFN (top panel), the four chemotherapeutic drugs (middle panel), and the chemotherapeutic drugs each with 0.01 µg/ml of both rHuIFN-α and rHuIFN-γ (bottom panel) on mesothelioma cell line M9K.

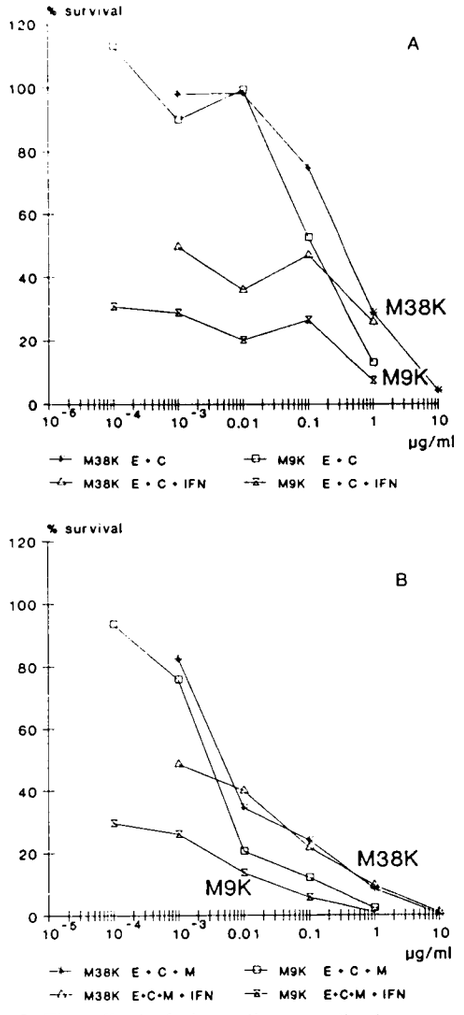

Figure 3. The effect of chemotherapeutic drug combinations, with and without 0.01µg/ml of both rHuIFN-α and rHuIFN-γ, on two mesothelioma cell lines. (A) Etoposide (E) and cisplatin (C) in combination with rHuIFN-α and rHuIFN-γ. (B) Etoposide, cisplatin and mitoxantrone (M) in combination with rHuIFN-α and rHuIFN-γ.

associated with a significant degree of cardiotoxicity, whereas mitoxantrone causes less cardiotoxicity.[25]

Combinations of drugs which show some activity individually against mesothelioma (e.g. cisplatin and doxorubicin) have been tested clinically but with little improvement in response.[3,4] Our results using drug combinations seem to support this observation. Early enthusiasm for combinations of etoposide and cisplatin in the treatment of small cell lung cancer and refractory lymphomas[28,29] has not been supported by more recent studies.[30] Interest has rather been focused on high dose single agent therapies, involving, in particular, etoposide and cisplatin.[24,31,32]

The slight difference in TC_{50} values for M38K between the two series of experiments may be due to the longer treatment time in the second series, as may also be the greater variation in other experimental results.[33]

Cell line M9K, from a patient previously treated with mitoxantrone (total dose 96 mg) and radiotherapy (70 Gy), was more sensitive to all the drugs and to IFN than was the primary tumor cell line, M38K. This suggests that the original therapy may have modulated the tumor cell response. Tumor cells from treated patients often show increased resistance to therapeutic agents.[33,34] We found that much less platinum was bound to the cellular DNA of the tumor cells than to the cellular DNA of the normal fibroblasts used as controls. It has been shown that the incidence of cisplatin–DNA adducts in human subjects undergoing cisplatin-based therapy increases as a function of platinum dose and, further, that the clinical response to therapy correlates well with the level of adduct formation (for review, see Greene[35]). Our finding of reduced DNA binding of platinum in the tumor cells may well reflect a decreased response of

Table 2. The platinum concentration in the cellular DNA in the three mesothelioma cell lines and in HFF cells. (For the tumor cell lines, the platinum concentration detected is also expressed as the percentage of the amount bound to the normal cells.)

Cisplatin treatment (µg/ml)	Platinum concentration detected (ng/mg DNA)				
	experiment 1		experiment 2		
	HFF	M33K	HFF	M14K	M38K
Control	ND[a]	ND[a]	30	23	16
0.3	28	17 (61%)	47	26 (55%)	21 (45%)
3.0	755	289 (38%)	227	62 (27%)	51 (23%)
15.0	3270	1930 (59%)	1695	653 (39%)	285 (17%)

[a] ND, not detected.

the original tumors to cisplatin therapy. It has been suggested that multidrug resistance involves an increased rate of efflux of the drug from the resistant cells.[34] We assume that this is one of the possible mechanisms behind the ineffective binding of cisplatin observed in these cell lines.

There are many recent reports in the literature of IFN-α and IFN-γ augmenting the effects of chemotherapeutic drugs, both *in vitro* and *in vivo*.[14,15,36] Sklarin *et al.*[13] reported that rIFN-α improved the sensitivity of malignant mesothelioma xenografts to mitomycin and/or cisplatin. The addition of IFN to the drug treatments in our study improved the mesothelioma cells' response to all the drugs and drug combinations. This suggests that IFN was having a general effect on the cells, e.g. by facilitating the access of the drugs to the interior of the cells through changes in membrane permeability.[34] Our experience was that all our mesothelioma cell lines would respond to IFN-α, but only some would respond to IFN-γ.[21] Other workers have also reported the variable sensitivity of mesothelioma cell lines to IFN-γ.[37,38]

There have been recent reports of the successful intrapleural treatment of malignant mesothelioma in the early stages with rIFN-γ.[39] Maintenance therapy with IFN following tumor reduction by other means is proving successful in the treatment of other malignancies.[11,15,19] The diagnosis of malignant mesothelioma at an early stage is crucial for the development of therapy because of the difficulties associated with the treatment of extensive disease. Not only can extensive disease be impossible to resect; the effectiveness of subsequent therapy is much reduced when there is a large tumor burden.

We conclude that our *in vitro* findings, taken in the context of clinical experience,[7,14,37] suggest that further studies of mitoxantrone, 4-epirubicin and IFN should be undertaken in the development of mesothelioma therapy.

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