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Enhanced effectiveness of last generation antiblastic compounds vs. cisplatin on malignant pleural mesothelioma cell lines

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Abstract

The purpose of this study was to examine the antiproliferative potentialities of a pool of new generation compounds (Paclitaxel, Docetaxel, Gemcitabine, Topotecan, SN-38) together with fenretinide, a synthetic derivative of retinoic acid, in comparison with the current first choice treatment cisplatin molecule, on a pool of human malignant pleural mesothelioma cell lines derived from either bioptic and pleural effusions samples. To evaluate the chemosensitivity features of malignant mesothelioma cells in vitro, we resorted to a rapid and reproducible colorimetric assay, a useful widely recognized tool for preclinical drug screening. In addition, by DNA content analysis and cellular morphologic assessment, we focused on the apoptosis as a potential mechanism of drug activity. The main results clearly indicate that, in all the models of malignant mesothelioma we handled in vitro, each tested antineoplastic agent is more powerful than cisplatin in inhibiting cell proliferation. Moreover, on experimental evidences basis, we can assume that the cytotoxic activity of tested compounds could be related, at least partially, to the drug-induced programmed cell death. This experimental study gives substance to the expected pharmacologic worth of the second generation antineoplastic drugs even if, in order to afford the most satisfactory biopharmacological approach, allowing to bypass the refractoriness to chemotherapy of this highly lethal tumour, further investigations at preclinical level are required.

Keywords: Malignant mesothelioma; Experimental therapeutics; Antiproliferative activity; Apoptosis

1. Introduction

Malignant mesothelioma, recognized in humans in the late 19th century (Lubrasch, 1895), is a rare, aggressive and invariably lethal tumour which originates from mesodermal-derived tissues of serosal cavities. It has been demonstrated by epidemiological studies that the occurrence of mesothelioma is strongly linked with prior asbestos exposure. It appears that the crocidolite variety of asbestos fiber type, classifiable as a complete carcinogen, produces sufficient alterations in normal mesothelial cells to generate a malignant population (Mossman et al., 1996); similar cases are not seen after amosite and chrysotile exposure (Britton, 2002). These modifications are presumably due to direct interaction of asbestos fibers (Jensen et al., 1996; Yegles et al., 1993) and by the onset of an inflammatory environment containing strong proliferative signals as well as active oxygen species (Dong et al., 1994; Liu et al., 1997). Three histological subtypes of the disease have been described: epithelial, sarcomatous and mixed histologies; unfortunately, the difficulties in establishing a definite histopathologic diagnosis, due to the variable clinical presentation, result in significant delays in treatment (Zellos and Sugarbaker, 2002). Although asbestos is indicated as the environmental factor most commonly associated with mesothelioma, not all mesotheliomas are associated to asbestos exposure and only a minority of people exposed to asbestos develop mesothelioma (Testa et al., 1998); moreover, asbestos does not transform human mesothelial cells in culture: these indications suggested that additional carcino-

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gens could act in concert with asbestos to cause mesothelioma. In 1993, Carbone first reported simian virus 40 (SV40) able to induce pleural mesotheliomas in hamsters (Cicala et al., 1993); later, the same author showed that 60% of 48 human mesotheliomas studied contained SV40-like DNA sequences (Carbone et al., 1994) implying that an SV40-like virus may act as independent pathogenetic factor or as co-carcinogen with asbestos. It has been demonstrated that the oncogenic activity of SV40 is due to the large T-antigen (Tag), encoded by the early genomic viral sequences. SV40 Tag retains ability to bind and to inactivate several cellular proteins such as p53 (Carbone et al., 1997) and pRb. T antigen causes knockout of p53 activity followed by cell arrest in G1 phase or apoptosis; furthermore, its binding to pRb provokes tumour formation. In addition, it seems that SV40 Tag protein is able to induce the expression of the highly mitogenic polypeptide type I insulin-like growth factor, random chromosomal alterations, genic rearrangements and point mutations. Two out of four mesothelioma cell lines established in our laboratory and used in the present study were found strongly positive for intranuclear Tag protein, but only one between the two virus-infected has been derived from a previously asbestos-exposed patient (Orengo et al., 1999). In the last decade, many evidences indicated that the pathogenesis of mesothelioma could depend on a possible synergy between SV40 infection and asbestos exposure.

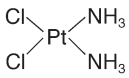
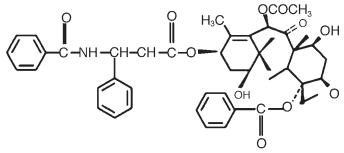
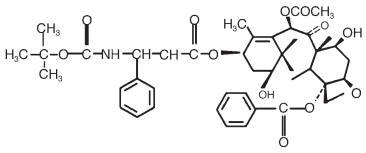
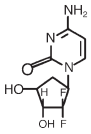
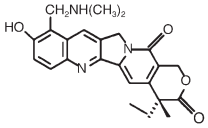
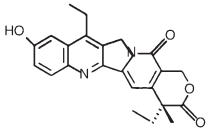
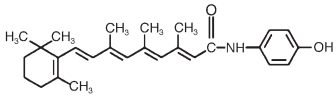
The incidence of malignant mesothelioma is rising, and it is expected to continue to increase into the next decade, particularly in developed regions with mining and shipyard industries (Britton, 2002). Furthermore, much higher incidence has been shown in men, thus implying occupational rather than environmental exposure (Peto et al., 1999). Unfortunately, malignant mesothelioma is a neoplasm for which standard multimodality regimens of treatment, based on surgery, chemotherapy and radiation, have no impact on median survival (approximately 7–9 months after diagnosis) (Aisner, 1995; Ong and Vogelzang, 1996) or, to the best, result in improved survival only in properly selected patients. Therefore, innovative therapies are still needed to prolong survival in patients with early and advanced disease (Zellos and Sugarbaker, 2002). Although nearly every class of cytotoxic agent has been evaluated in mesothelioma, response rates of greater than 20% have not been consistently demonstrated for any drug. The alchilant cisplatin (Table 1), and other platinums drug classes, which is conventionally employed in standard clinical chemotherapeutic protocols, together with antracyclines (doxorubicin), the antibiotic mitomycin and high dose of antifolates (methotrexate), is among the antineoplastic agents that have demonstrated some activity against malignant mesothelioma and has historically been considered the gold-standard agent (Baas, 2002; Fizazi et al., 2002; Girling et al., 2002; Kindler, 2000; Mintzer et al., 1985; Ong and Vogelzang, 1996; Zidar et al., 1988). In addition, combined chemotherapy does not appear to provide better results than single agent (Ardizzoni et al., 1991; Chahinian et al., 1993; Kindler, 2000; Ong and Vogelzang, 1996). Nevertheless, since 1991, Bowman and colleagues found mesothelioma cell lines intrinsically resistant to cisplatin and to other seven common antineoplastic drugs. They also found those cells resistant to tumour necrosis factor but displaying varying degrees of sensitivity to interferons α and γ (Bowman et al., 1991). Thus, it clearly appears that, in order to improve the clinical outcome in the pharmacological treatment of this refractory tumour, new drugs directed against novel tumour-specific cellular targets and/or characterized by a more specific mechanism of action are needed. More recently, four new chemotherapeutic agents have been found potent inhibitors, at various degree, of mesothelioma cell growth in vitro (Ollikainen et al., 2000). In a feasibility study (Knuuttila et al., 2000) on 15 previously untreated patients, CPT-11 and Docetaxel in combination could not be recommended, but their use, individually or in combination with cisplatin, has been warranted. McLaren et al. (2000) described different degrees of sensitivity of mesothelioma cell lines to new and traditional agents. The in vivo therapeutic efficacy of taxol tested in nude mice carrying subcutaneous mesothelioma has also been demonstrated (Min Lee et al., 1995).

Among the most strategic subcellular targets for anticancer compounds are the microtubules. Unlike classical agents as the vinca alkaloids, which induce microtubules depolymerization, taxanes are a class of antimitotic natural molecules, including Paclitaxel and Docetaxel (Table 1), able to enhance microtubule assembly and to inhibit the depolymerization of tubulin as well. Thus, normal mitotic spindle formation and subsequent cell division are blocked (Gelmon, 1994). In addition, it has been demonstrated that Paclitaxel is able to induce tumour necrosis factor-α (TNF-α) gene expression (Burkhart et al., 1994).

Gemcitabine (2′,2′-difluoro-2′-deoxycytidine) (Table 1) is an anticancer nucleoside, analog of deoxycytidine, which shares structural and metabolic similarities with 1-β-D-arabinosyl-cytosine (Ara-C). Inhibition of DNA synthesis is the major property of the antimetabolite Gemcitabine. In cells, this compound is converted to its triphosphate which is incorporated into DNA and terminates DNA strand elongation. Following this step, at the time that one additional deoxynucleotide is incorporated, the DNA polymerase is unable to proceed (“masked chain termination” process). Furthermore, Gemcitabine counteracts DNA synthesis indirectly by inhibiting ribonucleotide reductase (Plunkett et al., 1995). Gemcitabine has been shown to be more active in combination with a platinating agent than as a single agent. A single phase II study reported an impressive 48% in response rate for the combination Gemcitabine–cisplatin (Kindler, 2000).

Camptothecin, with its derivatives Topotecan (Table 1) and CPT-11, exert their antineoplastic action by inhibiting the 100 kDa nuclear enzyme topoisomerase I activity (Houghton et al., 1993; Slichenmayer et al., 1993). In particular, the covalent adduct between DNA and topoisomerase I is stabilized by these compounds. CPT-11 is a pro-

Table 1
Traditional and "last generation" antiproliferative compounds

Drug	Commercial name	Chemical name	Class	Structural formula	Empirical formula	M.W.
Cisplatin	Platinex	*cis-diamino-dicloro Pt*	alchilant		$Pt(NH_3)_2Cl_2$	300
Paclitaxel	Taxol	*Taxus brevifolia derivative*	antimitotic		$C_{47}H_{51}NO_{14}$	853.9
Docetaxel	Taxotere	*Taxus baccata derivative*	antimitotic		$C_{43}H_{53}NO_{14}$	807.89
Gemcitabine	Gemzar	*2',2'-difluoro-2'-deoxycytidine*	antimetabolite		$HClC_9H_{12}ClF_2N_3O_4$	299.66
Topotecan	Hycamtin	*9dimethylaminomethyl-10-hydroxy-camptothecin*	topoisomerase-I inhibitor		$C_{23}H_{23}N_3O_5ClH$	457.9
CPT-11 (SN-38)	Irinotecan	*(4S)-11-ethyl-camptothecin-9*	topoisomerase-I inhibitor		$C_{22}H_{20}N_2O_5$	392.41
4-HPR	Fenretinide	*N-(4-hydroxyphenyl) retinamide*	apoptosis, inducer, growth factors down-regulator		$C_{26}H_{30}NO_2$	391.26

drug that, differently from Topotecan, requires a de-esterification in vivo to yield SN-38 (Table 1), a metabolite 1000-fold more potent than the parental compound (Wiseman and Markham, 1996).

The antiproliferative effects of retinoids, a group of natural and synthetic vitamin A derivatives, are mediated by two classes of nuclear receptors, the retinoic acid receptors and the retinoid X receptors, which are members of the steroid–thyroid hormone receptor superfamily (Leid et al., 1992). *N*-(4-hydroxyphenyl)retinamide (4-HPR) (Table 1), commercially named fenretinide, is a synthetic analog of retinoic acid which was found to induce apoptosis in various tumour cell lines (Favoni et al., 1998; Lotan, 1995). Differently from the above mentioned compounds, the agent 4-HPR, as best we know, has not been previously tested in mesotheliomas. Early trials of angiogenesis inhibitors, gene therapy and vaccines offer additional avenues for treatment (Kindler, 2000); novel agents, including ZD1839 (Iressa), an orally administered, highly selective inhibitor of epidermal growth factor receptor tyrosine-kinase, and STI-571 (Gleevec), a highly selective inhibitor of platelet-derived growth factor receptor tyrosine-kinase, are being tested in a phase II trial (Nowak et al., 2002; Smythe, 2002).

In an effort to identify drugs endowed with a higher therapeutic index than that of cisplatin, we have investigated the antiproliferative potentialities of some of the above-described antiblastic compounds on a panel of histologically heterogeneous human malignant pleural mesothelioma cell lines.

2. Materials and methods

2.1. Drugs and chemicals

Paclitaxel ($C_{47}H_{51}NO_{14}$; molecular weight = 853.9), purchased from Sigma (St. Louis, MO, USA), Docetaxel ($C_{43}H_{53}NO_{14}$; molecular weight = 807.89), provided by

Rhône-Poulenc Rorer, Centre de Recherche de Vitry-Alfortville, (Vitry sur Seine, Cedex, France) and 4-HPR ($C_{26}H_{30}NO_2$; molecular weight = 391.26), provided by RW Johnson Pharmaceutical Research Institute (Spring House, PA, USA) were dissolved in absolute ethanol and stored at −20 °C (taxanes) or −80 °C (retinoic acid derivative). Gemcitabine ($HClC_9H_{12}ClF_2N_3O_4$; molecular weight = 299.66), from Eli-Lilly, Lilly Corporate Center, Indianapolis, IN, USA, was prepared in double distilled water and stored at 4 °C as well as Topotecan ($C_{23}H_{23}N_3O_5ClH$; molecular weight = 457.9), obtained from SmithKline Beecham Pharmaceuticals (King of Prussia, PA, USA). The other camptothecin derivative compound SN-38 ($C_{22}H_{20}N_2O_5$; molecular weight = 392.41), provided by Rhône-Poulenc Rorer, was dissolved in dimethylsulfoxide and stored at 4 °C. *Cis*-diamminodichloroplatinum [$Pt(NH_3)_2Cl_2$; molecular weight = 300 (cisplatin, Bristol-Myers Squibb, Oncology Division, Princeton, NJ, USA)] was diluted in saline buffer and stored at 4 °C. In order to avoid photoisomerization, all procedures involving drugs were performed under subdued lighting. Tissue-culture media and Dulbecco's phosphate-buffered saline were from ICN (Milan, Italy), whereas the (3-[4,5-Di*me*thyl*t*hiazol-2-yl]-2,5-diphenyl*t*etrazolium bromide) (MTT) salt was purchased from Sigma.

2.2. Cell lines and growth conditions

Malignant mesothelioma cell lines used in this study were derived from both pleural effusions (IST-Mes1, IST-Mes2, IST-Mes3 and MPP89) and tumour bioptic samples (SPC212, ZL34 and ZL55) from untreated patients. In particular, IST-Mes1, IST-Mes2 and IST-Mes3 cell lines were established in our laboratory (Orengo et al., 1999); MPP89 cells were obtained from Dr. M. Ribotta (Acqui T., Italy), whereas SPC212, ZL34 and ZL55 cell lines were kindly provided by Dr. B. Wegemann and Prof. R.A. Stahel (Zurich, Switzerland). The SV40-transfected pseudo-normal mesothelioma cells, Met5A, were purchased from ATCC (Rockville, MD, USA). IST-Mes1, IST-Mes2 and IST-Mes3 cell lines were maintained in HAM'S-F10 medium supplemented with 10% heat-inactivated foetal calf serum (HI-FCS), glutamine (2 mM) and antibiotics (0.02 U/ml penicillin and 0.02 mg/ml streptomycin). The other malignant mesothelioma cells, as well as the pseudo-normal cells Met5A, were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented as above unless the HI-FCS was 5% and 1% non-essential aminoacids was added. Cells were periodically monitored for mycoplasma contamination using the Mycoplasma Detection kit (Boeringher-Mannheim, Germany). The histological subtype characteristics of all cell lines, together with other informations on their origin, are reported in Table 2.

2.3. Cell growth evaluation

On the basis of preliminary experiments, cells were plated in duplicate in 16 mm diameter multiwell plates at the density of 5,000–10,000 cells/ml of appropriate medium and allowed to attach overnight (37 °C and 5% CO_2). At days 1, 2, 3, 4, 5, 7, 9 and 11 after plating, cell monolayer from each well was harvested by trypsinization. Cells were counted in a hemocytometric chamber; the viability was evaluated by the trypan-blue dye exclusion method and then doubling-time was calculated.

2.4. Chemosensitivity evaluation

The cell response to drug treatment was evaluated with the MTT colorimetric assay. Because this method requires standardisation according to cell doubling time, the optimal cell density of each cell line was examined and selected by preliminary experiments. Exponentially growing cells were plated, in triplicate, in 96-well microtiter plates at a density

Table 2
Characteristics of patients with malignant mesothelioma and their derived cell cultures

Cell lines							Patients			
	Year[a]	Origin	Histological subtype	Medium	d.t. (h)	Passage[b]	Sex	Age	Asbestos exposure	Previous chemotherapy
IST–Mes1	1996	pleural effusion	epithelial	HAM'S-F10 + 10% FCS	58	32–65	F	70	–	–
IST–Mes2	1996		epithelial		42	38–74	M	65	+	–
IST–Mes3	1996		mixed		87	34–61	M	81	+	–
MPP89	1989		epithelial		37	65–98	M	67	+	–
SPC212	1992	bioptic sample	mixed	DENM + 5% FCS	45	18–61	F	47	+	–
ZL34	1992		fibrous		33	21–74	M	53	+	–
ZL55	1992		epithelial		55	12–50	M	52	+	–
Met5A[c]	1989	SV40 transfected	epithelial		58	20–65	un.	un.	–	–

d.t. doubling time.
un. unknown.

[a] Year of in vitro cells establishment (Orengo et al., 1999; Schmitter et al., 1992; Ke et al., 1989).
[b] Range of culture passages at which the experimental study has been performed.
[c] Met5A are normal mesothelial cells derived from pleural fluid and immortalized by SV40 early region genes transfection.

of $1-6 \times 10^3$ cells/well in 200 μl of culture medium. Following overnight incubation, spent medium was removed and replaced with fresh medium containing the appropriate concentrations of the compounds under study. In particular, we used concentrations ranging from 0.01 to 40 μM of Paclitaxel, Docetaxel, Gemcitabine, Topotecan and SN-38 (Loprevite et al., 1999), ranging from 1 to 10 μM of 4-HPR (Favoni et al., 1998) and from 0.1 to 100 μM of cisplatin (Loprevite et al., 1999). After 48, 72, 96, 120 and 144 h of drug exposure, MTT assay was performed as previously described (de Cupis et al., 1995). The drug toxicity has been found not influenced by the maximum concentration % (v/v) of solvent (ethanol and dimethylsulfoxide) in culture media of both drug-treated and untreated groups.

In order to investigate whether drug treatment induced cytostatic or cytotoxic effect in our experimental model, cell growth recovery assay was performed. A selected panel of cell lines, one of pleural effusion origin (IST-Mes1), one derived from tumour bioptic sample (ZL34) and the SV40-transformed cells (Met5A), was used. Briefly, cells were seeded in triplicate, in 96-well microtiter plates, at the appropriate density, in 200 μl of culture medium. Following an overnight incubation, cells were exposed to Paclitaxel (drug representative of taxanes category), Gemcitabine, Topotecan (drug representative of camptothecin derivatives), cisplatin and 4-HPR. A couple of drug amounts were selected on the basis of the chemosensitivity assays and according to several exploratory experiments. After 72 h, drug-containing medium was removed and replaced with drug-free medium for additional 48 and 72 h of cell growth. At the end of each period, MTT assay was performed as mentioned above.

2.5. Morphologic assessment

We evaluated whether drug exposure modifies cellular morphology. Briefly, IST-Mes1, ZL34 and Met5A cells were plated in duplicate in 35-mm diameter Petri dishes in 2 ml of appropriate medium at the density of $1 \times 10^5 - 1.5 \times 10^5$ cells/well and incubated overnight. Cells were then treated with [IC_{50}] of Paclitaxel, Gemcitabine, Topotecan, cisplatin and 4-HPR. When untreated monolayers approached 70% confluence (48 or 72 h after drug exposure), treated and untreated cells were fixed and stained according to the May–Grunwald/Giemsa method and then examined with a LEICA DMIRB inverted-phase microscope (Germany). Photographs were then taken with a JVC 3CCD camera and elaborated by a Mac G3 computer using the Image Pro-Plus imaging software (Media Cybernetics, USA).

2.6. DNA content analysis by flow cytometry

IST-Mes1 and ZL34 malignant mesothelioma cell lines and the pseudo-normal Met5A cells were plated in 60-mm diameter Petri dishes and incubated overnight (37 °C, 5% CO_2). Cells were then treated with the [IC_{50}] of Paclitaxel (representative of taxanes), Gemcitabine, Topotecan (representative of camptothecin-derivative), cisplatin and 4-HPR for 48 and 72 h. At the end of each incubation period, cells were harvested and fixed in cold 70% ethanol and extensively washed with phosphate-buffered saline. Samples were incubated for 6 h with the nuclei staining solution, containing propidium iodide (40 μg/ml) in the presence of ribonuclease. Treated and untreated cells were analyzed using a Coulter XL flow cytometer; histograms were acquired in linear mode and then analyzed using the Multicycle DNA software (Phoenix Flow Systems, San Diego). The presence of hypodiploid peak has been considered representative of apoptotic cells (Darzynkiewicz et al., 1992; Nicoletti et al., 1991).

In order to detect apoptotic cells, an Annexin V FITC (fluorescein isothiocyanate) Kit (Bender MedSystems, Vienna, Austria) was employed as described (Martin et al., 1995) on the selected ZL34 cell line. To this end, after 48 and 72 h treatment with Paclitaxel, Gemcitabine and 4-HPR, cells were harvested, washed with phosphate-buffered saline, stained with Annexin V-FITC solution and then with the non-vital dye propidium iodide. The percentage of Annexin V positive (early apoptotic cells) as well as Annexin V and propidium iodide positive cells (late apoptotic or necrotic cells) were calculated.

2.7. Statistical analysis

Results are reported as mean percentages ± standard error (S.E.) of three experiments performed in duplicate (cell count) or in triplicate (MTT assay). Statistical significance between treated and untreated groups was evaluated by Wilcoxon test. SPSS program (for Windows) has been used for the [IC_{50}] values determination using Probit Statistical Analysis.

3. Results

3.1. Cell growth evaluation

Cell proliferation studies revealed that both malignant mesothelioma cell lines and SV40-transformed Met5A cells reach the exponential growth phase within 2–7 days after plating. Doubling times of the cells under study appear to be quite heterogeneous, ranging between 33 and 87 h, specifically, 33 h (ZL34), 37 h (MPP89), 42 h (IST-Mes2), 45 h (SPC212), 55 h (ZL55), 58 h (IST-Mes1 and Met5A) and 87 h (IST-Mes3) (Table 2).

3.2. Responsiveness to drug treatment

The antiproliferative potentialities of the novel generation compounds vs. the conventional drug *cis*-diamino-dichloroplatinum (cisplatin) were evaluated on malignant mesothelioma cell lines, derived from either pleural effusion and surgical specimens, by the colorimetric MTT assay.

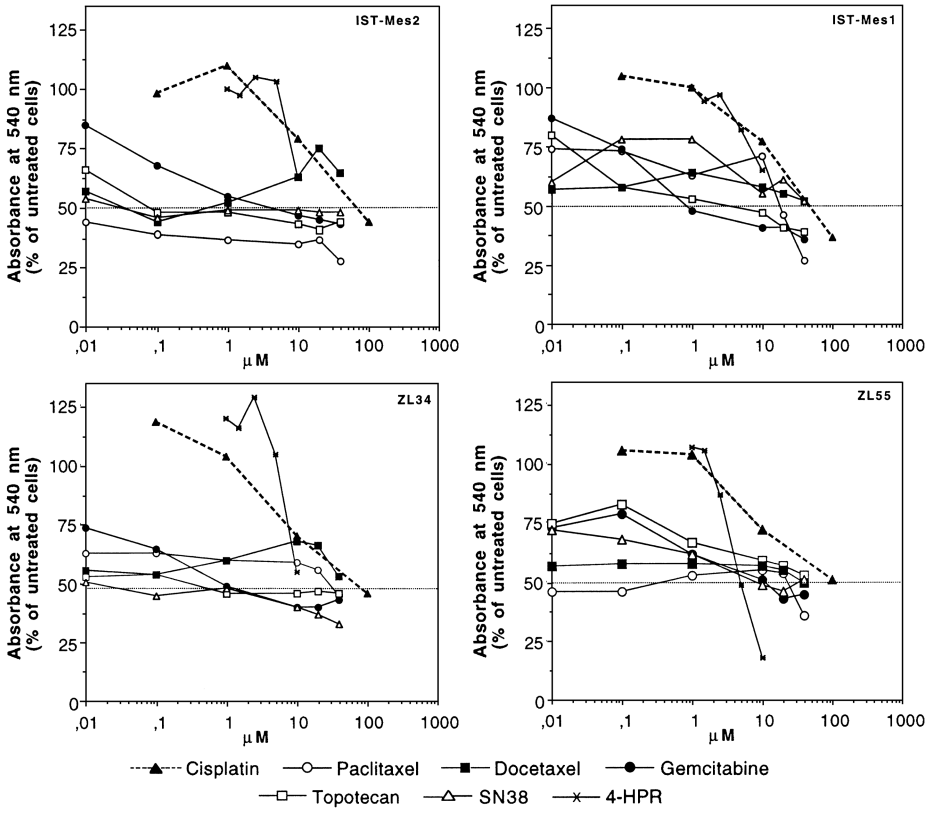


Fig. 1. Diagrams are representative of, respectively, the most chemosensitive (IST-Mes2 and ZL34) and most drug-resistant (IST-Mes1 and ZL55) pleural effusion and bioptic samples-derived cells lines. Each curve, which depicts the mean of three experiments performed in triplicate, shows cellular chemosensitivity at time exposure, detailed in the text, at which each drug was able to induce the 50% inhibition of cell growth. Standard errors are too small (<4%) to be graphically represented.

Average data of each triplet of experiments were plotted and, from concentration–response diagrams as well as from the data process by a specific statistical program, the amount of drug able to halve proliferating cells, [IC_{50}], was calculated. Three arbitrary [IC_{50}] ranges, in order to discriminate the cell high sensitivity (0.01 μM–1 μM), medium sensitivity (1 μM–10 μM) and low sensitivity-resistance (10–100 μM) to the tested drugs, have been adopted. Collectively, data from represented concentration–response curves provide the following informations: (a) all cell lines examined show, in a concentration-dependent manner, low sensitivity to cisplatin and not much sensitivity to 4-HPR (Fig. 1 and Table 3); (b) IST-Mes2, IST-Mes3 and ZL34 show the highest sensitivity (0.01–1 μM) to all drugs (Table 3,

Table 3
Chemosensitivity of malignant mesothelioma cell lines evaluated by the colorimetric MTT assay and expressed by IC_{50} [μM][a]

origin	Cell lines	*Taxanes*		*Antimetabolites*	*Campothecin-derivative*		*Fenretinide*
		Cisplatin	Paclitaxel / Docetaxel	Gemcitabine	Topotecan / SN-38		4-HPR
pleural effusion	IST–Mes1	50 (4)	20 (5) / >40 (6)	0.8 (4)	3 (4) / 40 (4)		10 (5)
	IST–Mes2	70 (4)	0.01 (6) / 0.03 (5)	4 (4)	0.07 (4) / 0.03 (5)		10 (4)
	IST–Mes3	50 (5)	40 (4) / 0.01 (4)	1 (4)	10 (4) / 0.1 (4)		(b) (b)
	MPP89	100 (4)	10 (5) / 1 (5)	40 (4)	10 (4) / 1 (4)		5 (6)
biopsy	SPC212	100 (2)	40 (2) / 0.01 (3)	1 (2)	10 (2) / 10 (2)		10 (2)
	ZL34	70 (2)	30 (2) / 0.01 (2)	1 (2)	0.5 (2) / 0.03 (2)		≈10 (2)
	ZL55	100 (3)	0.01 (3) / 0.01 (3)	10 (3)	40 (3) / 10 (3)		5 (4)
SV40 transf.	Met5A	100 (2)	40 (2) / 0.01 (4)	0.01 (3)	1 (3) / 0.01 (3)		10 (2)

( ) in brackets, days of drug exposure at which the [IC_{50}] has been reached.

[a] [IC_{50}] values reported in this table are those able to inhibit 50%±5 cellular proliferation and each value represents the mean of three experiments performed in triplicate.

[b] Not reached [IC_{50}] even at the highest concentration [10 μM] and the longest time of drug treatment (6 days).

shaded areas) but to Gemcitabine, Paclitaxel–Topotecan and Paclitaxel, respectively: these three cell lines are very sensitive to the wider range of drugs (three to four out of six) at the lower concentrations (0.01–1 μM); (c) IST-Mes1, MPP89, SPC212 and ZL55 show the lowest sensitivity (10–100 μM) but to Gemcitabine, Docetaxel-SN38, Docetaxel–Gemcitabine and Paclitaxel–Docetaxel, respectively: on these cell lines, only few drugs are active at concentrations of 1 μM or less (Table 3, shaded areas); (d) most of the responses to drugs, but to cisplatin and 4-HPR, especially for the very chemosensitive IST-Mes2 and ZL34 cell lines, are concentration-independent reaching or approaching the maximal effect around 50% inhibition of cell proliferation already at the lowest concentration tested (Fig. 1 and Table 3).

Times at which cell lines reached 50% of growth inhibition are detailed, in round brackets, in Table 3. [IC_{50}] are reached in a range of 2–6 days of drug exposure showing variability among cell lines and a time-dependent response to the pharmacological treatment. In general, cell lines derived from biopsy (SPC212, ZL34 and ZL55) reached [IC_{50}] in a shorter time (2–3 days) with respect to pleural effusion-derived cells (IST-Mes1, IST-Mes2, IST-Mes3 and MPP89) which needed a double time of drug exposure, 4–6 days, to be 50% growth (Table 3). The Met-5A SV40-transfected cells are sensitive, with different level of sensitivity, to the same drugs—Docetaxel, Gemcitabine and both the camptothecin-derivative—than the biopsy-derived ZL-34 cell line, but, with respect to this last, the [IC_{50}] is reached after a longer time of drug treatment (3–4 days), resembling the behavior of pleural-derived cells (Table 3). Taken together, the group of four pleural effusion-derived cell lines is as much chemosensitive as the group of three biopsy-derived cells, while the pseudo-normal Met5A cells appear very sensitive almost like the ZL34 cell line. Important differences in drug activity have been shown among the different classes of molecules tested in this study: Docetaxel is more effective than Paclitaxel in all cell lines, apart from IST-Mes1 which is low-sensitive to taxanes, whereas SN-38 is lightly better than the other camptothecin-derived Topotecan. Furthermore, all six novel generation drugs are more effective, in certain cases consistently, than the traditionally used cisplatin; among them, 4-HPR is the least effective for the models tested. Finally, dissimilarities in drug activity (2–3 logs) were observed among cell lines vs. the same compound: the most active, in terms of [IC_{50}], resulted Docetaxel and SN-38, whereas the worse appeared 4-HPR and cisplatin.

In Fig. 1, are reported dose–response "S" curves representative of the most chemosensitive IST-Mes2 and ZL34 (left panels), respectively pleural effusion and bioptic samples-derived cells lines, and the couple of the same respective derivation but less chemosensitive IST-Mes1 and ZL55 (right panels), as evinced by the MTT assays. In detail, IST-Mes2 cellular proliferation resulted 50% inhibited by <0.1 μM of Topotecan after 96 h of exposure, whereas it only required less than half concentration of SN-38, together with 24 additional hours, to obtain the same effect. Four days of treatment was also able to halve IST-Mes2 growth when cells were exposed to Gemcitabine ($IC_{50}=4$ μM), 4-HPR ($IC_{50}=10$ μM) and cisplatin ($IC_{50}=70$ μM). The 50% reduction of proliferating cells was obtained at the lower concentration used (0.01 μM) for Paclitaxel and at 0.03 μM for Docetaxel after 144 and 120 h, respectively. Concerning ZL34 cell line (Fig. 1), the camptothecin-derivatives, Topotecan and SN-38, and Docetaxel were the most potent agents, showing an [IC_{50}] almost 10^4 log lower than cisplatin. After 2 days of treatment, 50% of cell proliferation

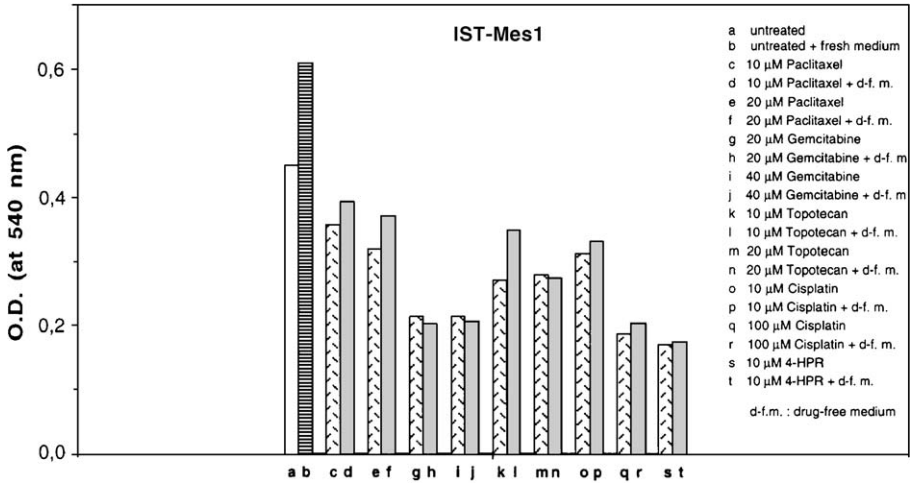
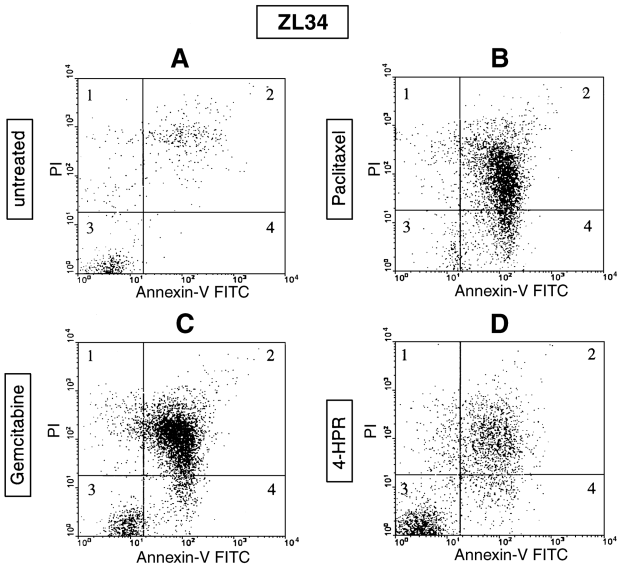


Fig. 2. Cell growth recovery evaluated by MTT assay on IST-Mes1 cell line after 72 h of drug treatment followed by 72 h of culture in fresh drug-free medium (d-f. m.). Columns represent the mean (standard errors are not graphically clearly representable) of three independent experiments performed in triplicate. The non-parametric Wilcoxon test revealed that differences between 72 h drug-treated and untreated cells were significant ($0.001<P<0.004$), whereas differences between 72 h drug-treated cells followed by 72 h of culture in d-f. m. were not significant. Open column: untreated cells; lined column: untreated cells + fresh medium; chipped columns: drug-treated cells; shaded columns: drug-treated cells + fresh medium.

inhibition was reached by 4-HPR and Paclitaxel at higher concentrations (10 and 30 μM, respectively); between the compounds belonging to taxanes class, Docetaxel appeared to be 3000-fold more potent than Paclitaxel. Finally, the deoxycytidine analog Gemcitabine was able to halve proliferating cells at the concentration of 1 μM. On the IST-Mes1 cell line, after a 4-day treatment, Gemcitabine appeared as a potent drug [IC_{50}] = 0.8 μM (Fig. 1). Cellular proliferation was also halved after 96 h of exposure by Topotecan and SN-38, the first showing an [IC_{50}] more than 10-fold lower than the other camptothecin-derivative (IC_{50} = 3 and 40 μM, respectively). After the same time of treatment, it has been revealed that cisplatin was able to decrease cell growth by 50% at 50 μM. With respect to taxanes, it was found that Paclitaxel [IC_{50}] was 20 μM at 120 h and that one additional day together with 2-fold higher concentration was required to inhibit proliferation of Docetaxel-treated cells by 50%. Five days of 4-HPR exposure at the highest concentration used (10 μM) were necessary to halve proliferating IST-Mes1 cells (Fig. 1). MTT assay performed on ZL55 cell line (Fig. 1) revealed that, after 72 h of treatment, 100 μM cisplatin halved viable cells, whereas 10^4-fold lower concentration (0.01 μM) of Paclitaxel and Docetaxel was able to induce the same antiproliferative behavior. On the other hand, the maximal concentration tested (40 μM) of Topotecan was required to reach the 50% inhibition. ZL55 cellular proliferation appeared to be more affected by the other compound belonging to the camptothecin-derivative class, SN-38, as well as by Gemcitabine (IC_{50} = 10 μM). One additional day of exposure (96 h) was required to decrease by 50% cell growth by 5 μM 4-HPR.

In order to establish whether the antiproliferative effect of the tested compounds was reversible upon their removal, MTT experiments of cell growth recovery were performed on a selected panel of cell lines, as described in Materials and methods. Upon drugs withdrawal, ZL34, IST-Mes1 as well as Met5A cell lines were not able to acquire again proliferative activity by 48 and 72 h in a drug-free medium culture, indicating that the inhibitory effect of the compounds used, independently from their mechanism of action, was cytotoxic rather than cytostatic. In Fig. 2, the most representative IST-Mes1 cell line, after 72 h in a drug -free medium, is shown.



panel	A	B	C	D
area	untreated	Paclitaxel [30 μM]a	Gemcitabine [1 μM]a	4-HPR [10 μM]a
1	9.43	13.1	14.8	14.1
2	4	37.1	44.5	13.2
3	81.4	12.9	25	63
4	5.2	36.9	15.7	9.72
2 + 4	**9.2**	**74.0**	**60.2**	**22.92**

a) in brackets drug concentration, corresponding to [IC_{50}], used for 72 h drug exposure

Fig. 3. Flow cytometry analysis of ZL34 malignant mesothelioma cell line. Cells, before staining with fluorescein isothiocyanate (FITC)-labelled Annexin V and propidium iodide, were cultivated for 72 h in the absence (panel A) or in the presence of Paclitaxel (panel B), Gemcitabine (panel C) and 4-HPR (panel D). Clusters: 1 = necrotic cells (propidium iodide positive); 2 = late apoptotic and necrotic cells (propidium iodide/Annexin V positive); 3 = intact living cells (propidium iodide/Annexin V negative); 4 = early apoptotic cells (Annexin V positive). These values are reported as percentage in the table, where the 2 + 4 areas represent the total of necrotic + apoptotic cells.

3.3. Drug-induced apoptosis

The measurement of Annexin V binding was performed in association with propidium iodide to discriminate and quantitate between apoptotic and necrotic cells on the ZL34 cell line (Fig. 3). [IC_{50}] of Paclitaxel and Gemcitabine (30 and 1 μM, respectively) after 72-h treatment (panels B and C, area 4) showed a significantly high percentage of apoptotic cells vs. untreated cells (panel A, area 4). 4-HPR-treated cells showed a percentage of apoptotic cells, with respect to the two other drugs, four and half time lower, respectively (panel D, area 4). Clusters showing necrotic + apoptotic cells as well as apoptotic cells alone (areas 2 and 4, respectively) confirm the higher capacity to induce apoptosis of Paclitaxel followed by Gemcitabine and 4-HPR on the examined ZL34 cell line.

3.4. DNA content analysis

Appearance of fractional DNA content, typical of apoptotic cells (Darzynkiewicz et al., 1992), was found by flow cytometry analysis in ZL34 cell line following 48 h of exposure to 30 μM Paclitaxel (24% apoptotic cells), to 10 μM 4-HPR (12%) and to 1 μM Gemcitabine (10%). A lesser

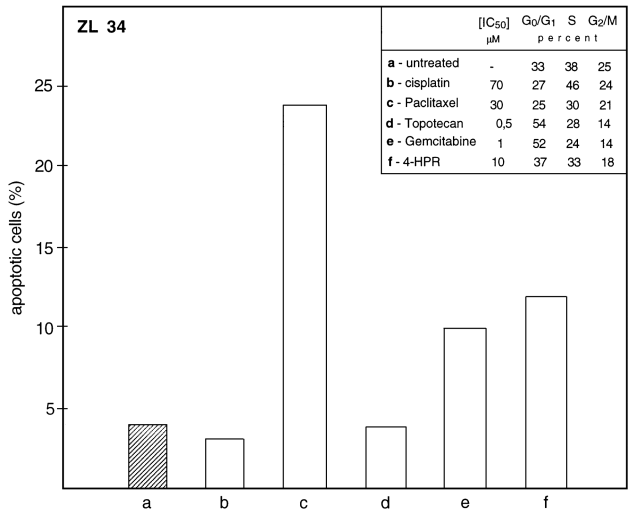


Fig. 4. Apoptotic cell population of ZL34 malignant mesothelioma cell line, after 48-h drug treatment. Inset: percentage distribution of DNA content among the cell cycle phases of ZL34 cells after 48-h drug exposure measured by flow cytometry.

apoptotic population (≈ 4%), comparable to that of untreated cells, was evident after Topotecan and cisplatin treatment (0.01 and 100 μM, respectively) (Fig. 4). An accumulation in the G2/M phase (≈ 48%), in the absence of apoptotic cells, was detectable only in Paclitaxel-treated (10 μM for 72 h) IST-Mes1 cell line, whereas fractional DNA content (range 16–27%) was observed after exposure (72 h) to the other compounds used (data not shown). DNA analysis performed on Met5A cells revealed that all drugs under investigation were able to induce apoptosis, without affecting cell progression through and/or accumulation in any cell cycle phase. Particularly, exposure to the retinoic acid derivative 4-HPR (10 μM for 48 h) caused an ≈ 48% DNA fragmentation, whereas treatment with cisplatin (100 μM, 48 h) and Topotecan (1 μM, 72 h) led to an ≈ 30% of apoptotic cells and the amount of fractional DNA in Paclitaxel (40 μM, 48 h) and Gemcitabine (0.01 μM) treated cells was ≈ 20% (data not shown).

3.5. Cellular morphology

Cellular morphology, evaluated at 70% confluence of untreated cells, showed that IST-Mes1 cells, revealing their epithelial origin, grew as cobblestone-like mats (Fig. 5A), whereas ZL34 cells grew as spindle-shaped (Fig. 5D). In Fig. 5, also shown are the morphological changes exhibited by IST-Mes1 and ZL34 after exposure to the corresponding [IC_{50}] of Topotecan and 4-HPR (IST-Mes1: Fig. 5B and C), Paclitaxel and Gemcitabine (ZL34: Fig. 5E and F). The antiproliferative and cytotoxic action of the compounds used strongly decreased the cell numbers, as evinced by the loss of cell monolayer, in comparison to untreated cells. Furthermore, drugs treatment was able to cause cell shrinkage, nuclear condensation and rupture of the cells into debris.

4. Discussion

The increasing incidence of malignant mesothelioma in industrialized world together with the failure of conventional chemotherapeutic regimens (median survival ≈ 1 year) have made the search for new drugs and novel approaches of critical and mandatory importance. A large number of

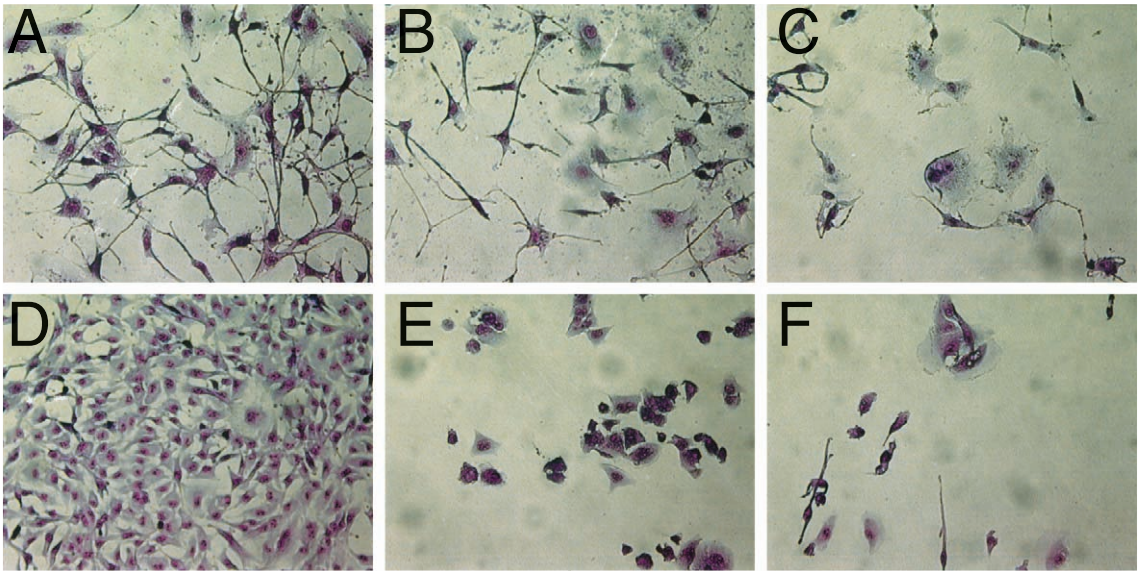


Fig. 5. Morphological changes induced on IST-Mes1 and ZL34 cell lines by the exposure to the [IC_{50}] of several compounds representative of the four classes of drug screened. Treated and untreated cells were fixed and stained, as described, when the monolayers of the untreated cells approached 70% confluence. (A) Untreated IST-Mes1 cells; (B) Topotecan-treated (3 μM, 72 h) IST-Mes1 cells; (C) 4-HPR-treated (10 μM, 72 h) IST-Mes1 cells; (D) untreated ZL34 cells; (E) Paclitaxel-treated (30 μM, 48 h) ZL34 cells; (F) Gemcitabine-treated (1 μM, 48 h) ZL34 cells. Morphological alterations observed are typical of cells undergoing apoptosis. Photographs were taken at 20 ×.

natural and synthetic compounds have been developed and tested, for their antiproliferative properties, in tumours which had previously been considered to be as resistant to chemotherapy as malignant mesothelioma, like non-small cell lung cancer (Loprevite et al., 1999) and breast carcinoma (Favoni et al., 1998). Unfortunately, the identification of more effective pharmacological treatment for malignant mesothelioma appears difficult because, due to the lower incidence of this neoplasm, the number of patients eligible for clinical evaluation of more advanced molecules is relatively small (Singhal and Kaise, 2002). In addition, the limited advances in highlighting the biology of this tumour, as well as in testing new antiproliferative agents, are also due to the paucity of well-characterized human cell lines and practical animal models. A number of studies (Craighead and Mossman, 1982; Topov and Kolev, 1987) reported induction of mesothelioma in several animal species (e.g. rats and rabbits) by intrapleural injections and inhalation of asbestos. These in vivo models are certainly relevant for understanding carcinogenesis and tumour development. Nevertheless, their utility in searching for an effective pharmacological treatment for mesothelioma in humans is limited by the unreliability of tumour induction, the long latency period of this disease and the differences existing among species. Thus, the availability of established human malignant mesothelioma cell lines provides a rapid, practical and less expensive solution to investigate the basic biology of this malignancy and to seek out novel therapeutic approaches. That said, in this study, we evaluate the in vitro antineoplastic potentialities of taxanes, Gemcitabine, camptothecin derivatives and 4-HPR, typical representative of a so-called last generation of anticancer compounds endowed with different mechanisms of action, on a selected panel of human malignant mesothelioma cell lines.

The aim of our investigation was to compare the antiproliferative activity of these molecules with that of cisplatin and derivatives which, at present, represent the best standard pharmacological treatment for this aggressive tumour. In addition to the systematic preclinical screening of the compounds used, in order to better clarify the chemosensitivity characteristics of the cell lines under investigation, drug-induced modulation on DNA content and its effect on cellular morphology were also analyzed.

The most interesting finding of this study is that all compounds tested out resulted more potent than cisplatin in inhibiting malignant mesothelioma cell lines proliferation, independently of their origin and histological subtype. Moreover, the drug treatment resulted was also effective in counteracting cell growth of the SV40-transformed Met5A cells, thus showing no significative differences between malignant and these pseudo-normal mesothelioma cells. To our knowledge, this is the first report comparing the in vitro antiproliferative activity of the listed novel agents with that of the conventional compound cisplatin on malignant mesothelioma cells. Nevertheless, we have recently demonstrated on non-small cell lung cancer cell lines (Loprevite et al., 1999) a superimposable behavior of these drugs. Even McLaren et al. (2000) who investigated the action of taxanes, Gemcitabine, camptothecin derivatives and vinca alkaloids on a panel of malignant mesothelioma cell lines in terms of inhibition of cellular proliferation and modulation of interleukin-6 secretion demonstrated a similar drug behavior. While the reduced effectiveness of cisplatin, as previously mentioned, has been observed in all cell lines studied, our results indicate pharmacological variability among the novel chemotherapeutic agents. Thus, at least in our experimental model, it is not possible to identify, among the new molecules, a drug that could be preferred to another for promotion to clinical trial. In particular, we observed that Docetaxel appears more potent than Paclitaxel in six out of eight cell lines. On the other hand, the activity of both taxanes does not seem to be concentration-dependent. This independence from the concentrations used in inhibiting cellular proliferation is not surprising since it has been already reported in other in vitro models (Hill et al., 1994; Loprevite et al., 1999). Differently from our findings, McLaren and colleagues (McLaren et al., 2000) described, using our same experimental approach but a different complement of mesothelioma cell lines, a relative insensitivity of their cell lines to both Paclitaxel and Docetaxel. The results obtained by these authors appear to be in agreement with those of clinical studies (Caliandro et al., 1997); nevertheless, we would underline that the discrepancies of these findings could just be related to possible differences between ours and their in vitro model (e.g. intrinsic resistance to taxanes) and the low number of phase II studies. Furthermore, we found that Paclitaxel was able to induce apoptosis in two (ZL34 and Met5A) out of three cell lines examined, as demonstrated by DNA content analysis and typical morphological changes. Thus, the cytotoxic effect of Paclitaxel, demonstrated by the cell inability to recover proliferation after drug withdrawal, could be related to the programmed cell death induced by treatment. These findings are in agreement with those reported by Olah et al. (1996) who analyzed the molecular mechanisms involved in the antiproliferative action of Paclitaxel in breast, ovarian and leukemia cell cultures. On the other hand, analysis of cell cycle revealed a block in the G2/M phase of Paclitaxel-treated IST-Mes1 cell line, and this result is in accordance with the peculiar mechanism of action of this drug. Regarding the camptothecin-derivative compounds, no significative differences were noted between Topotecan and SN-38 (the active metabolite of CPT-11) in inhibiting cellular proliferation. These findings are similar to those previously obtained in our laboratory on a panel of non-small cell lung cancer cell lines (Loprevite et al., 1999). In addition, mesothelioma cells chemosensitivity to these compounds has also been demonstrated by McLaren et al. (2000). As postulated for taxanes, the cytotoxic effects of Topotecan could be due, at least partially, to the drug-induced apoptosis. Recently, a pilot phase II clinical trial has indicated that the combination of cisplatin and CPT-11 had a definite activity against malignant pleural mesothelioma

(Nakano et al., 1999). As described in Results, the deoxycytidine analog Gemcitabine resulted able to down-regulate cellular proliferation of both malignant and pseudo-normal mesothelioma cells. Similar findings were obtained by McLaren et al. (2000) on their in vitro models. The Gemcitabine-induced apoptosis is a phenomenon already described, even though on other experimental models (Huang and Plunkett, 1995). In particular, it has been proposed that the DNA inhibition synthesis due to the drug incorporation into the nucleic acid, which therefore resulted damaged, could probably activate a signalling pathway resulting in cell death by apoptosis (Plunkett et al., 1995). At present, our experimental results do not appear completely confirmed by clinical trials. In particular, van Meerbeeck et al. (1999) have published that single-agent Gemcitabine therapy demonstrated only limited therapeutic activity on malignant mesothelioma at the schedule and dosage that commonly are employed in untreated patients. Using the same schedule, Bischoff et al. (1998) reported a 30% response rate. The combination of Gemcitabine and cisplatin has been found synergistic against mesothelioma cell lines in vitro; the combination with cisplatin or carboplatin shows definite activity in phase II trials (Kindler and van Meerbeeck, 2002). Future roles of Gemcitabine in malignant mesothelioma patients include Gemcitabine/platinum regimen for neoadjuvant or adjuvant therapy combining it with other cytotoxic agents, such as Pemetrexed or Vinorelbine, or cytostatic agents such as vascular endothelial growth factor inhibitors (Kindler and van Meerbeeck, 2002). The retinoic acid derivative exerted its antiproliferative activity only at the highest concentration used, whereas at the lowest, we were unable to evidence any significative cell growth modulation. These results, which to our knowledge are the first regarding 4-HPR treatment on malignant mesothelioma cells, do not concur with those previously obtained in our laboratory on breast cancer cell lines. In that study, using both the MTT as well as the thymidine incorporation assays, we demonstrated that breast carcinoma cells resulted more sensitive to 4-HPR treatment, even at a concentration of 1 μM (Favoni et al., 1998). Furthermore, it has been demonstrated (Zou et al., 1998) that fenretinide inhibits cellular proliferation of lung cancer cell lines. Nevertheless, in agreement with our (Favoni et al., 1998) and other (Lotan, 1995; Oridate et al., 1996; Ponzoni et al., 1995) previous findings obtained using different experimental model, we observed 4-HPR-mediated programmed cell death. The concentration able to induce apoptosis (10 μM) in our experimental model is higher (≈ 10-fold) than the peak plasma level in patients receiving 4-HPR (200 mg/daily). This observation could be relevant in vivo since it has been reported that some tissue can concentrate retinoid, as evidenced by the presence of >10 μM in the nipple discharge of a woman who had taken oral 4-HPR for 15 days (Formelli et al., 1993).

In summary, our findings show that the second generation of anticancer agents tested in this study seems to be more potent than cisplatin, the typical representative of platinum-derived compounds, in inhibiting malignant mesothelioma cells proliferation. Combinations of the individually tested agents with platinum-derived compounds and other molecules, such as Pemetrexed disodium (Alimta), Raltitrexed (Tomudex), ZD1839 (Iressa) and STI571 (Gleevec), have already been planned. The results presented in this paper, which warrant to be confirmed and deepened by further investigations, appear promising and relevant especially considering the high refractoriness of this tumour to the conventional therapeutic regimens. The validation in clinical trials of innovative standard-based protocols will help to prolong survival in patients with early and advanced disease.

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