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Mitozolomide activity on human cancer cells *in vitro*

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Summary The growth inhibitory effects, the reduction of [^{3}H]-TdR incorporation and the perturbation of the cell cycle induced by the new agent mitozolomide on the M14 human melanoma cell line and on the SW626 human ovarian cancer cell line were compared to those produced by BCNU. Flow cytometry showed an interesting difference: at the high concentration mitozolomide induced an accumulation of cells in S middle and S late-G2-M phase of the cell cycle whereas BCNU caused only a block in S late-G2-M. Further studies were aimed at investigating the susceptibility of freshly isolated human ovarian cancer cells to pharmacologically reasonable mitozolomide concentrations. Only in one out of 16 primary cultures of human ovarian cancers was mitozolomide able to induce cell cycle perturbation, suggesting that ovarian carcinoma cells may not be sensitive to this drug.

Mitozolomide (M), (NSC 353451) (Stevens *et al.*, 1984) or 8-carbamoyl-3-(2-chloroethyl)-imidazo-[5, 1-d]-1, 2, 3, 5-tetrazin-4(3H)-one, is a new anticancer agent which has shown striking activity against rodent tumors (Hickman *et al.*, 1985). Its mechanism of action appears related to the formation of DNA interstrand-cross-links (DNA-ISC) in a manner similar to that described for chloroethylnitrosoureas (Gibson *et al.*, 1984*a*, *b*).

Like the chloroethylnitrosoureas, M does not produce DNA-ISC in cells which are able to remove the crosslinkable monoadducts bound to O^6 of guanine (i.e. cells with MER^+ phenotype) (Gibson *et al.*, 1984*b*). On the other hand, in contrast to the most commonly used chloroethylnitrosoureas (e.g. BCNU), M shows no carbamylating activity (Stevens *et al.*, 1984; Horgan & Tisdale, 1984) and this may perhaps explain some of the differences in its pharmacological effects.

In this study we compared the antiproliferative effects and the perturbation of the cell cycle produced by M and BCNU on the human melanoma cell line, M14, and on the ovarian carcinoma cell line, SW626, and investigated the effects of M on 16 primary cultures of human ovarian cancer cells freshly isolated from patients.

Materials and methods

SW626, a human ovarian cancer cell line (Fogh *et al.*, 1977*a*, *b*), was grown at 37°C in air plus 5% CO_2 in RPMI 1640 supplemented with 10% FCS, 2 mM L-glutamine (Gibco Europe, Glasgow, UK), 10 mM $NaHCO_3$, buffered with HEPES 20 mM (Merck, Darmstadt, W. Germany).

The human melanoma cell line M14 (Golub *et al.*, 1976), was also grown in RPMI 1640 but without HEPES.

The effects of M were studied in primary cultures derived from 6 primary ovarian adenocarcinomas, 2 omental metastases and 8 ascitic fluids from 13 patients. The patients' main characteristics are shown in Table I. In patient no. 9 two paracenteses were performed at intervals of one month. Tumour biopsy specimens were collected in PBS containing 100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin (Gibco Europe, Glasgow, UK). Within 3 h of primary surgery, tumour tissue fragments were disaggregated by treatment with a 0.3% collagenase solution (Collagenase type 1, Sigma Chemical Company, St. Louis, USA) for 40 min at 37°C under continuous stirring. Cell suspension was centrifuged, washed in PBS then resuspended in growth medium. Tumour cell suspensions contaminated by RBC and/or leucocytes were further processed as for ascitic fluids.

The ascitic fluids were collected in heparinized bottles and the cells were separated by centrifugation. A first gradient with 100% of Ficoll-Hypaque (d=1.077; MSL, Eurobio, Paris) was performed (600 g for 20 min) to remove RBC contamination and debris. In cases of gross lymphocyte and granulocyte contamination, a second discontinuous gradient (75% Ficoll-Hypaque, layered on 100% Ficoll-Hypaque) was performed. After these steps, in all cases, tumour cells were freed of macrophages by adhesion on plastic culture dishes. Final cell suspensions,

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Table I Patients' characteristics

Pt. No.	*Age*	*PS*	*FIGO staging*	*Histology*	*Histological differentiation*	*Previous chemotherapy*
1	50	100	III	Serous	Poorly differentiated	None
2	68	80	III	Serous	Poorly differentiated	None
3	54	90	III	Serous	Poorly differentiated	None
4	63	90	III	Serous	Moderately differentiated	None
5	57	100	III	Serous	Poorly differentiated	None
6	57	70	IV	Endometrioid	Poorly differentiated	None
7	62	90	III	Serous	Poorly differentiated	None
8	50	80	III	Serous	Moderately differentiated	DDP
9	60	80	III	Serous	Moderately differentiated	PAC
10	52	90	III	Serous	Poorly differentiated	PAC
11	60	80	III	Serous	Poorly differeintiated	PAC
12	62	90	III	Mucinous	Poorly differentiated	PAC
13	47	100	III	Serous	Poorly differentiated	PAC

DDP = *cis*-dichlorodiammineplatinum.
PAC = *cis*-dichlorodiammineplatinum + cyclophosphamide + doxorubicin.

containing more than 70% viable cells (erythrosine dye test) were seeded at 70,000 cells cm^{-2} in 24-well multiwell tissue culture plates (Falcon, Becton Dickinson Labware, Oxnard, CA, USA) (Morasca *et al.*, 1983). The medium for human tumour cells was the same as that for SW626 cells.

For the *in vitro* treatment, M (kindly supplied by Prof. M.F.G. Stevens, Aston University, Birmingham, UK or by Dr C.G. Newton, May and Baker Ltd, Dagenham, Essex, UK) was dissolved in medium plus dimethylsulfoxide (DMSO) to obtain a maximal concentration of 0.0025% DMSO, which is not toxic to the cells, and left in contact with the cells for 24 h; BCNU (Kindly supplied by the Division of Cancer Treatment, NCI, Bethesda, Md, USA) was dissolved in ethanol to obtain a maximal concentration of 1% and left in contact with the cells for 24 h. After that cultures were drained, washed in PBS and filled with fresh growth medium for 72 h (recovery time). The cytotoxic effects of M and BCNU were evaluated after treatment and/or recovery by two different methods:

(a) as inhibition of thymidine [^{3}H]-TdR incorporation, adding 0.5 μCi [^{3}H]-TdR sp. act. 1.9 Ci mM^{-1} (Schwarz Mann, Orangeburg) to the well for the last hour of treatment or recovery time. At the end of incubation cells were washed twice with PBS, lysed by 1% sodium dodecylsulfate (SDS) and counted in a toluene-based phosphorus scintillation fluid with a Packard Tricarb 3400 scintillator;

(b) as a reduction in the cell count, using a Coulter Counter model ZB (Coulter Electronics, Ltd, UK).

Controls and each treatment group comprised 8–10 replicate cultures. Statistical analysis was done by Dunnett's test using a Hewlett-Packard 85 computer (Colombo *et al.*, 1986).

Flow cytometry studies were performed on a 30L cytofluorograph (Ortho Instruments, US). Cells in culture were washed with PBS after drug treatment or recovery and directly stained with 2 ml of propidium iodide (PI) solution containing 50 $\mu g\,ml^{-1}$ PI (Calbiochem Behring Co, USA) in 0.1% sodium citrate, 25 μl 1% nonidet P40 as detergent (Sigma) and 25 μl RNAse 0.5 $\mu g\,ml^{-1}$ in water (Calbiochem Behring Co, USA) at room temperature for 30–40 min. With this method nuclei were dislodged from cells adhering to the plastic surface of the 24-well multiwell and entered into suspension without the cells having to be suspended (Colombo *et al.*, 1986).

The fluorescence pulse was detected in a spectral range between 580 and 750 nm. The coefficient of variation (CV) of the G1 peak of the cells was 3–4%.

Each cytofluorimetric assay was performed with $2–3 \times 10^5$ cells, and the percentage of the cell cycle phases was calculated by the method of Krishan & Frei (1976).

To determine the DNA index, human leucoytes from freshly collected blood were used as standard. The standard was run before and after the sample to check for drifting of the laser output. The CV of the leucocytes was between 1.5–2.5%. Ploidy was expressed as DNA index, representing the ratio between the G1 peak of ovarian cancer cells and the G0/G1 peak of leucocytes (Erba *et al.*, 1985).

Results

Figures 1 and 2 illustrate the effects of 24 h treatment with M and BCNU on M14 human

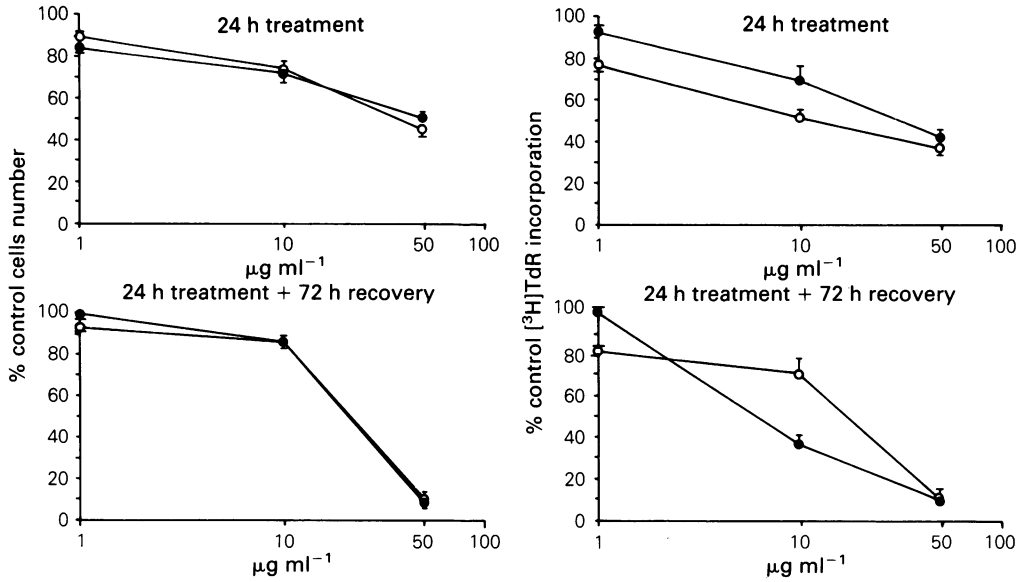

Figure 1 Effects of M (●) and BCNU (○) after 24 h treatment at concentrations of 1, 10 and 50 $\mu g\,ml^{-1}$ on M14 human melanoma cell line, evaluated as reduction in the number of cells (left panel) and as inhibition of [^{3}H]-TdR incorporation (right panel). Each value is the mean (±s.e.) of 8 replications.

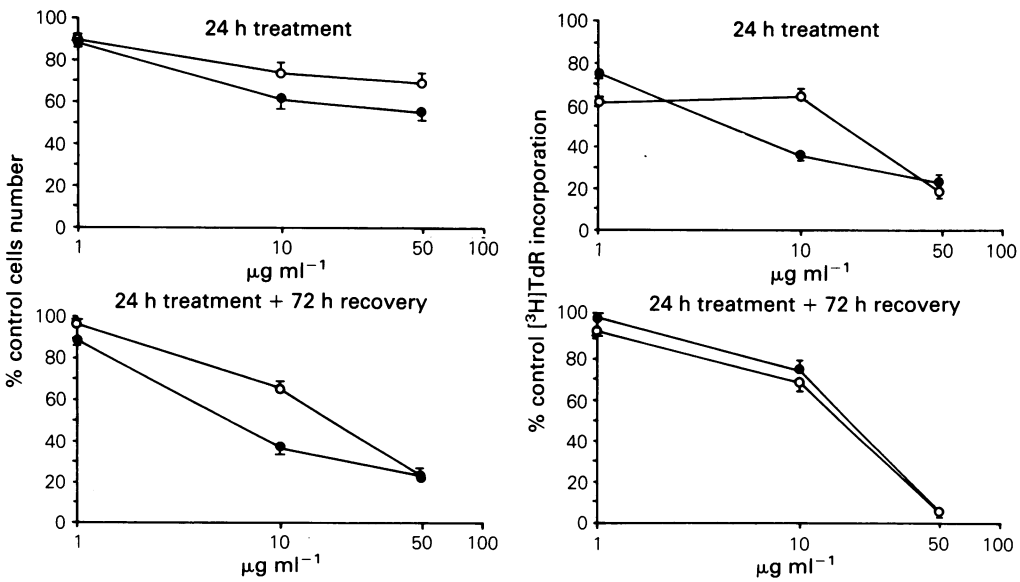

Figure 2 Effects of M (●) and BCNU (○) after 24 h treatment at concentrations of 1, 10 and 50 $\mu g\,ml^{-1}$ on SW626 human ovarian cancer cell line evaluated as reduction in the number of cells (left panel) and as inhibition of [^{3}H]-TdR incorporation (right panel). Each value is the mean (±s.e.) of 8 replications.

melanoma cells and on SW626 human ovarian cancer cells. In M14 cells M and BCNU caused an overlapping inhibition of cell growth and only slight differences in the [^{3}H]-TdR incorporation.

In SW626 cells M appeared moderately more effective than BCNU. M caused greater cell growth inhibition, the difference being statistically significant ($P<0.01$) 72 h after drug treatment with 10 μg ml^{-1}. On both cell lines 50 μg ml^{-1} M or BCNU caused very similar inhibition of cell growth and [^{3}H]-TdR incorporation and these effects were in fact stronger after 72 h recovery time.

The effects of M and BCNU on the cell cycle of M14 and SW626 were assessed by the flow cytometric technique (Figures 3 and 4). Both drugs caused an accumulation of cells in premitotic phase. This effect was only slight at 1 μg ml^{-1}, clear at 10 μg ml^{-1} and very marked at 50 μg ml^{-1}. At the

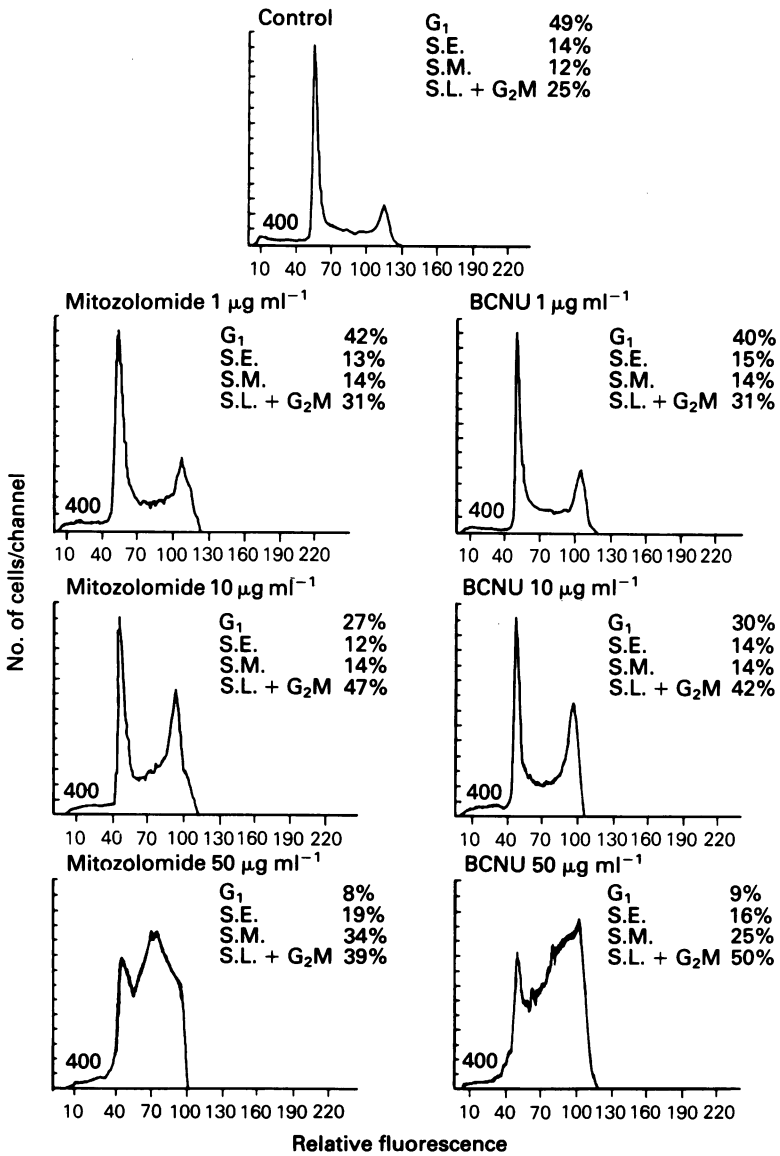

Figure 3 Effects of 24 h M and BCNU treatment at concentrations of 1, 10 and 50 μg ml^{-1} on cell cycle phase distribution in M14 human melanoma cell line.

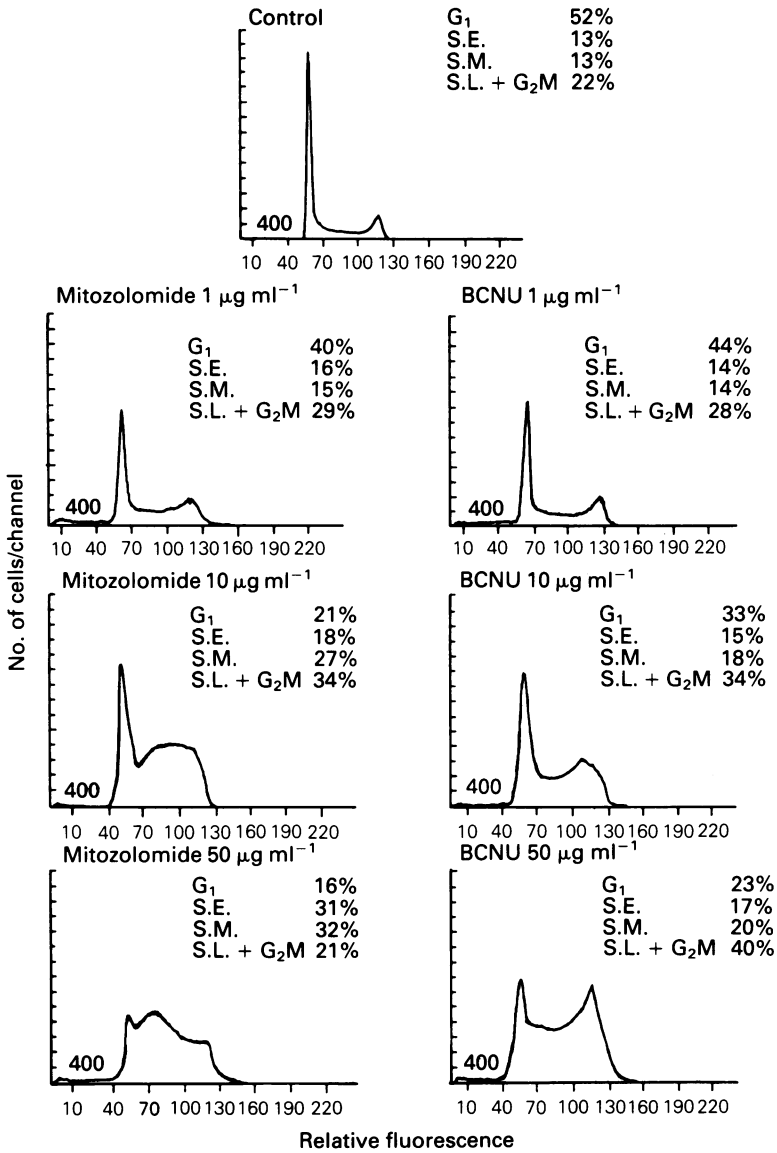

Figure 4 Effects of 24 h M and BCNU treatment at concentrations of 1, 10 and 50 $\mu g\,ml^{-1}$ on cell cycle phase distribution in SW626 human ovarian cancer cell line.

highest concentration M induced an accumulation of cells in SM and SLG2M, whereas BCNU caused an arrest only in SLG2M.

The activity of M was tested on 16 primary cultures of human ovarian cancer cells from 13 patients. Tables II and III show the results of flow cytometry analysis in ovarian cancer cells, derived from previously untreated or treated patients, after 24 h M treatment. When possible [^{3}H]-TdR incorporation after 24 h drug treatment was also evaluated. Only in case no. 3 was the cell cycle perturbed, with an accumulation of cells in the SE and SM phases. The slow progression through the S phase was consistent with the marked reduction of [^{3}H]-TdR incorporation observed in this case ($P<0.01$ at 1 and 10 $\mu g\,ml^{-1}$ M concentrations).

Table II Effects of M on the distribution in G1, S early (SE), S middle (SM), and S late +G2+Mitosis (SLG2M) and on [^{3}H]-TdR incorporation of human ovarian cancer cells (growing in primary culture) derived from patients previously untreated with antineoplastic agents. Cells were treated for 24 h.

Pt. no.	*Sample*	*G1*	*SE*	*SM*	*SLG2M*	*Mitozolomide* $\mu g\,ml^{-1}$	*DNA index*	*[^{3}H]-TdR % of control*
1	Primary tumour	57	12	12	19	control	1.30	
		56	13	11	20	0.1		100
		45	18	12	25	1		100
		44	19	12	25	10		90
2	Ascitic fluid	64	4	7	25	control	1.20	
		78	44	3	15	0.1		85
		74	3	4	19	1		84
		70	4	5	21	10		70[a]
3	Metastasis	42	10	10	38	control	1.87	
		35	18	21	25	1		37[b]
		38	18	23	21	10		28[b]
4	Primary tumour	69	5	5	21	control	1.27	—
		71	5	6	18	1		
		68	5	5	22	10		
5	Primary tumour	55	12	11	22	control	1.47	—
		53	14	12	21	1		
		48	17	12	23	10		
6	Primary tumour	71	8	8	13	control	1.80	—
		71	8	8	11	1		
		72	7	9	12	10		
6a	Ascitic fluid	61	10	13	16	control	1.80	—
		58	9	15	18	0.1		
		54	15	15	16	1		
		57	10	13	20	10		
7	Primary tumour	36	18	16	30	control	1.00	
		42	16	16	26	1		
		34	19	16	31	10		

DNA index was determined as described in **Materials and methods.** Statistical analysis was by Dunnett's test. [a]$P<0.05$; [b]$P<0.01$.

Discussion

As previously reported on murine Lewis lung carcinoma *in vivo* (Broggini *et al.*, 1986) and *in vitro* (Horgan *et al.*, 1983) M causes an accumulation of cells in the S late+G2M phase of the cell cycle.

At a high concentration M, unlike BCNU, also produces an accumulation of cells in SM phase. This may be related to quantitative or qualitative differences in the binding of the two drugs to DNA and possibly to differences in DNA damage and repair. That the biochemical features of M and BCNU differ is not suprising considering that BCNU causes protein carbamylation whereas M does not (Hickman *et al.*, 1985). It is known that the formation of DNA-ISC after chloroethylnitrosoureas treatment involves a first rapid alkylation of O^{6}guanine (i.e. chloroethylation or hydroxyethylation) followed by a second reaction with a cytosine located on the opposite strand of DNA (Erickson *et al.*, 1980). If a cell synthesized O^{6}guanine alkyl transferase (MER$^+$ phenotype) the monoadducts on O^{6}guanine are removed, thus preventing the formation of DNA-ISC. In contrast, in a cell which is O^{6}guanine repair-deficient (MER$^-$ phenotype) the monoadduct is not removed and can form DNA-ISC. Both chloroethylnitrosoureas and M induced DNA-ISC appear related to their cytotoxicity (Gibson *et al.*, 1984*b*).

However some differences which may perhaps underlie the different perturbation in the cell cycle phases were suggested by Gibson *et al.* (1984*a*, *b*). They found that M formed DNA-ISC more slowly than chloroethylnitrosoureas. In addition M caused much greater differential cytotoxicity between O^{6}alkylguanine repair proficient (MER$^+$ phenotype) and deficient (MER$^-$ phenotype) cells, suggesting that it forms more O^{6}guanine adducts than BCNU.

Even though these differences are of biochemical

Table III Effects of M on the distribution in G1, S early (SE), S middle (SM), and S late +G2+Mitosis (SLG2M) and on [^{3}H]-TdR incorporation of human ovarian cancer cells (growing in primary culture) derived from patients previously treated with antineoplastic agents. Cells were treated for 24 h.

Pt. no.	*Sample*	*G1*	*SE*	*SM*	*SLG2M*	*Mitozolomide* $\mu g\,ml^{-1}$	*DNA index*	[^{3}*H*]-*TdR* *% of control*
8	Ascitic fluid	52	12	10	26	control	1.79	
		55	10	11	24	0.1		100
		53	10	11	26	1		100
		47	10	11	32	10		75[a]
9	Ascitic fluid	85	3	3	9	control	1.00	
		82	3	4	11	0.1		100
		84	4	3	9	1		100
		83	4	4	9	10		100
9a	Ascitic fluid	36	14	14	36	control	1.00	
		43	14	16	27	0.1		90
		36	15	17	32	1		82
		34	14	16	36	10		82
10	Ascitic fluid	35	13	20	22	control	1.57	
		30	14	22	34	0.1		100
		29	16	22	33	1		100
		28	22	27	23	10		100
11	Primary tumour	86	1	1	12	control	1.00	
		88	1	1	10	1		100
		83	2	1	14	10		76
11a	Metastasis	66	3	4	27	control	1.00	—
		66	2	4	28	1		
		65	2	4	29	10		
12	Ascitic fluid	67	7	7	20	control	0.90	—
		40	9	10	41	10		
13	Ascitic fluid	56	14	12	18	control	1.67	—
		55	14	13	18	0.1		
		55	13	12	20	1		
		51	15	15	19	10		

DNA index was determined as described in **Materials and methods.** Statistical analysis was by Dunnett's test. [a]$P<0.05$; [b]$P<0.01$.

interest, their relevance to possible contrasting pharmacological effects is questionable since in rodent tumours M and chloroethylnitrosoureas showed a similar spectrum of activity (Hickman *et al.*, 1985) and were cross resistant (Gibson, 1982). In the present studies the cytotoxicity of M and BCNU against M14 and SW626 human cell lines was also similar.

The indication arising during phase I clinical trials that M might be active against human ovarian carcinoma (Newlands *et al.*, 1985) prompted us to investigate whether human ovarian epithelial cancer cells from biopsies of primary tumours or metastases or from ascitic fluids were sensitive to this agent. Using flow cytometry analysis, a method with proven sensitivity in detecting antiproliferative properties of this drug, we assessed whether M caused cell cycle perturbation on 16 primary cultures of ovarian carcinoma from 13 patients. M was only effective in one case in which a moderate accumulation of cells in S phase was seen. We did not test drug concentrations higher than $10\,\mu g\,ml^{-1}$ since a plasma peak level of $7\,\mu g\,ml^{-1}$ M was reported after the dose of $250\,mg\,kg^{-1}$, i.e. higher than that recommended as safe for further clinical investigations (Newlands *et al.*, 1985).

Since none of the patients had previously received drugs related to M (i.e. triazenes or nitrosoureas) it may be excluded that resistance was induced by previous treatment, and it appears more likely that ovarian carcinoma cells are naturally non-susceptible to this drug.

Whether this lack of sensitivity is due to these cells' ability to remove O^{6}alkylguanine adducts from DNA or to other mechanisms is now being investigated in our laboratories.

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