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In vitro chemosensitivity testing of Fotemustine (S 10036), a new antitumor nitrosourea

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Summary. Fotemustine (S 10036) is a new anti-tumor nitrosourea characterized by a phosphonoalanine carrier group coupled to the nitrosourea moiety, which potentially increases the cellular penetration of the drug. Using human tumor cell lines, the activity of S 10036 was compared with that of the more established nitrosoureas BCNU and CCNU. Growth-inhibiting effects were evaluated by the [^{3}H]-thymidine incorporation test. In a panel of 12 human cancer cell lines [melanoma (4), ovary (2), head and neck (3), lung (1), bladder (1), breast (1)], the dose-response curves of S 10036 (0 – 100 μM) were similar to those obtained with equimolar concentrations of BCNU and CCNU; they indicated a moderately more marked effect for two and an equal effect for six melanoma cell lines with S 10036 as compared with BCNU. Moderate but significant synergistic combinations were obtained when S 10036 (0 – 80 μM) and CDDP (0 – 100 μM) or DTIC (250 – 6,500 μM) were combined in melanoma cell lines. In conclusion, the new nitrosourea S 10036 shows promising activity, particularly against human melanoma cell lines.

Introduction

S 10036, or (*N*-(chloro-2-ethyl)-*N*-nitrosoureido)-1-ethylphosphonate (Fotemustine), is a new antitumor nitrosourea containing a phosphonoalanine carrier group grafted to the nitrosourea group, which potentially increases the cellular penetration of the drug. In addition, Fotemustine presents an interesting *n*-octanol/water partition coefficient (log P = 1.25). This drug has a high experimental activity as defined by the NCI screening system. A recent phase I study [6] reported that measurable responses can be obtained in melanoma patients. Comparative experimental analysis of the respective mechanisms of action of Fotemustine and BCNU indicated certain differences between these drugs [10]. These clinical and experimental data prompted the present three-part study: (1) the cytostatic activity of Fotemustine was compared with that of the established nitrosoureas BCNU and CCNU on a panel of 12 human cancer cell lines of different tumor types; (2) the activity of Fotemustine against melanoma was studied in detail (eight different cell lines); and (3) the effects of combinations of Fotemustine with DTIC or cisplatin on melanoma cell lines were analyzed (the latter drug has previously been shown to be synergistic with BCNU and CCNU [3]).

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Materials and methods

Drugs

The nitrosoureas Fotemustine (mol. wt., 315.7 kDa), BCNU (mol. wt., 214 kDa) and CCNU (mol. wt., 233.7 kDa) were obtained from the Institut de Recherches Internationales Servier (Neuilly/Seine France); Fotemustine was supplied as a 50-mg/ml solution in 95% (v/v) ethanol. BCNU and CCNU were supplied as pure crystalline powders, which were dissolved in ethanol at a final concentration of 50 mg/ml. Cisplatin (CDDP) and Dacarbazine (DTIC) were obtained from routine commercial sources via the pharmacy of the hospital; they were diluted according to the manufacturer's specifications. All drugs were stored at −20° C and constituted the stock solutions. The day before use, drugs were diluted in the cell-culture medium to a concentration 50 times that required in the final experiment; dilution was carried out using ice in a dark environment, and the solutions were stored overnight at −20° C. Tritiated thymidine (52 Ci/mmol) was purchased from CEA (Paris, France).

Cell lines

The tumor types and origins of the cell lines used are listed in Table 1.

Experimental Conditions

Dulbecco's modified Eagle's medium (DMEM), E199 medium, glutamine and fetal bovine serum (FBS) were purchased from Gibco (Paisley, UK). Penicillin and streptomycin were from Meyrieux (Lyons, France). The cells were routinely cultured in a humidified incubator (Sanyo) at 37° C in an atmosphere of 8% CO_2 in air.

Experiments. The effects of Fotemustine, BCNU and CCNU (study I) were compared using cell lines CAL 1, 2, 4, 7, 9, 12, 18, 27, 33 and 43, 100 P3 and 647 V, as indicated in Table 1. Drug concentrations included 5 μg/ml (low), 10 μg/ml (medium) and 20 μg/ml (high) (see Table 2 for molar equivalents); the medium dose was chosen to match the achievable in vivo drug concentration

Table 1. Cell lines studied

Tumor type	Cell line	Origin
Melanoma	cal 1, cal 4, cal 7	Centre A. Lacassagne, Haematology and Oncology Department [2]
	cal 23, cal 24, cal 32, cal 41, cal 48 A	Centre A. Lacassagne, Haematology and Oncology Department
Breast cancer	cal 18	Centre A. Lacassagne, Haematology and Oncology Department [4]
Ovary	cal 2, cal 9, cal 43	Centre A. Lacassagne, Haematology and Oncology Department
Head and neck	cal 27, cal 33	Centre A. Lacassagne, Haematology and Oncology Department [5]
Lung cancer: Epitheliomas	cal 12	Centre A. Lacassagne, Haematology and Oncology Department
Adenocarcinoma	100 P3	Prof. G. Julliard, UCLA
Bladder cancer: Transitional carcinoma	647 V	Prof. G. Julliard, UCLA

Table 2. Molar equivalents of S 10036, BCNU and CCNU

Dose (μg/ml)	Drug (μ*M*):		
	S 10036	BCNU	CCNU
5	15.8	23.4	21.4
10	31.7	46.7	42.8
20	63.4	93.5	85.7

Table 3. Drug dose range used for each cell line in the drug-combination study

Cell line	DTIC (m*M*)		CDDP (μ*M*)		S 10036 (μ*M*)	
cal 4	2.47	4.94	9	90	30	80
cal 1	0.247	2.47	9	23	1	5
cal 48	2.47	6.35	3.3	14.5	30	60
cal 32	2.47	4,94	3.3	29	30	60
cal 24	2.47	4,94	0.9	33	30	80
cal 23	0.247	4,94	0.9	9	1	5

[1]. The drugs were simultaneously added to the culture medium and incubation was carried out for 150 min at 37° C in an atmosphere containing 8% CO_2.

For comparison of the effects of Fotemustine and BCNU on melanoma (study II), cell lines CAL 1, 4, 7, 23, 24, 32, 41 and 48 A were used. Drug concentrations were: 10 μ*M* (low), 30 μ*M* (medium) and 60 μ*M* (high). The drugs were simultaneously added to the culture medium and incubation was carried out for 150 min at 37° C in an atmosphere containing 8% CO_2.

The effect of Fotemustine in combination with CDDP or DTIC (study III) was also investigated. Cells were selected on the basis of the results of study II. There were six melanoma cell lines; including two sensitive lines (CAL 1 and 23), two lines of intermediate sensitivity (CAL 24 and 48) and two resistant lines (CAL 4 and 32). The detailed experimental conditions are described in Table 3.

Chemosensitivity testing. Two slightly different methods were used for chemosensitivity testing; both are related to that described by Volm et al. [11]. Method A was used for part I, and method B was used for parts II and III. Logarithmically growing cells were trypsinized and suspended in DMEM plus 2 m*M* glutamine 10% (v/v) FBS, 400 IU/ml penicillin, and 200 μg/ml streptomycin (D10). The cell concentration was adjusted to 10^6 cells/ml. For method A, the cell suspension was distributed in the wells of 96-well microtitration plates (Greiner, France) at $2-4 \times 10^3$ cells/well. For method B, the cells were distributed in the wells of 24-well plates (Falcon, Los Angeles, Calif.) at $1-1.5 \times 10^4$ cells/well. The number of cells per well was chosen so as to maintain exponential growth during the assay (4 days). After the cells had set for 48 h, the medium was replaced and drugs were added under the conditions described above. After cell exposure the drugs were removed, and the cells were washed with DMEM and refed with D10. At various times after drug removal (6 h = t 0,6 h + 24 h = t1... 6 h + $n \times 24$ h = tn), the plates were respectively washed with E 199 medium (Gibco, Paisley, UK) and the cells were incubated overnight at 37° C in E 199 plus 10% FBS and 2.5 μCi/ml [^{3}H]-thymidine. In method B only, plates were cooled (45 min at 4° C) to stop thymidine incorporation, the medium was removed, and the cells were rinsed three times with cold PBS. Then, in both method A and method B, trichloroacetic acid cold 5% (TCA) was added and cells were allowed to precipitate for 1 h at 0° C. For method A, the cell precipitate from each well was collected on glass-fiber filter using a Skatron cell harvester (Flow, Irvine, UK); following digestion of precipitated material with Lumasolve (Lumac) at 37° C for 2 h, the radioactivity of

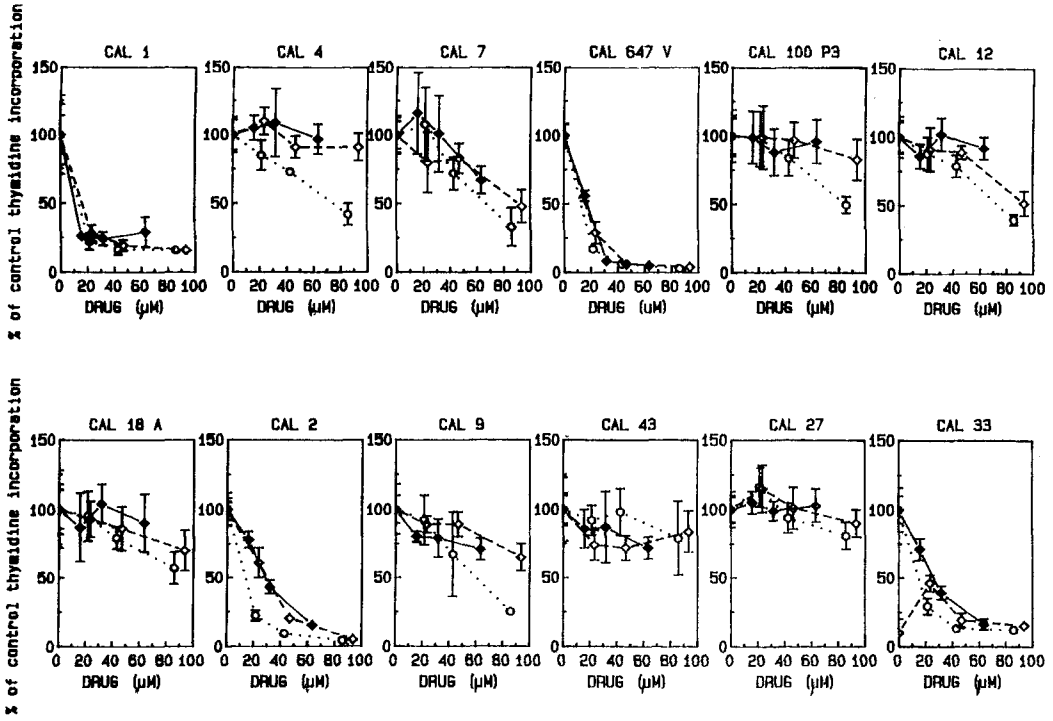

Fig. 1. Effects of three nitrosoureas on [^{3}H]-thymidine incorporation by 12 human tumor cell lines (drug concentrations expressed in μM). ◆ (*solid line*), S 10036; ◇(*dashed line*), BCNU; ○ (*dotted line*), CCNU. Time of contact with the drugs was 150 min. The assay was carried out 78 h after drug removal (*t3*)

Table 4. Chemosensitivity of 12 human tumor cell lines to S 10036, BCNU and CCNU

Cell line	S 10036	BCNU	CCNU
cal 1	S 24	S 19	S 16
cal 4	R 109	R 91	R 73
cal 7	R 101	R 83	R 72
647 V	S 8	S 6	S 6
100 P3	R 88	R 97	R 84
cal 12	R 102	R 89	R 79
cal 18 A	R 104	R 86	R 79
cal 2	S 43	S 20	S 9
cal 9	R 79	R 89	R 67
cal 43	R 87	R 72	R 98
cal 27	R 99	R 101	R 94
cal 33	S 39	S 19	S 13

Numbers indicate the RTI (relative thymidine incorporation compared with control values) for the intermediate dose at t3. For definitions and incubation conditions, see Materials and methods
R = resistant; S = sensitive

Table 5. Chemosensitivity of eight human melanoma cell lines to S 10036 and BCNU

Cell line	BCNU	S 10036
cal 1	VS 21	VS 16
cal 4	VR 110	VR 92
cal 7	VR 90	VR 107
cal 23	VS 16	VS 14
cal 24	VR 98	VR 84
cal 41	MS 59	MS 48
cal 32	VR 74	MS 66
cal 48	MS 51	S 28

Numbers represent the RTI (relative thymidine incorporation compared with control values) for an intermediate dose at t3. For definitions and incubation conditions, see Materials and methods
VR, very resistant; VS, very sensitive; MS, moderately sensitive; S, sensitive

the filter was counted. For method B, the cell precipitate was dissolved with 1 *N* NaOH; after neutralization by HCl and the addition of scintillation fluid (Dynagel; Baker Chemical, Daventer, Holland), the radioactivity of each well was counted on a Packard Tricarb liquid scintillation counter.

Results were expressed as the percentage of radioactive incorporation relative to control values (RTI); each result corresponds to the mean ± SD of six measurements. Cell sensitivity was evaluated as follows: (a) in experiment I, designed only to screen the cell lines for sensitivity to the drugs, a cell line was considered to be resistant (R) if RTI > 50% for the intermediate dose at t3 and sensitive (S) if RTI < 50% for the intermediate dose at t3; and (b) in experiment II, a cell line was considered, to be sensitive (S) if RTI (+ 1 SD) < 50% for the intermediate dose at t3, moderately sensitive (MS) if RTI < 50% <RTI + 1 SD or RTI > 50% > RTI − 1 SD for the intermediate dose at t3, resistant (R) if RTI − 1 SD > 50% for the intermediate dose at t3, very sensitive (VS) if RTI < 50% for the low dose at t3, and very resistant (VR) if RTI > 50% for the high dose at t3.

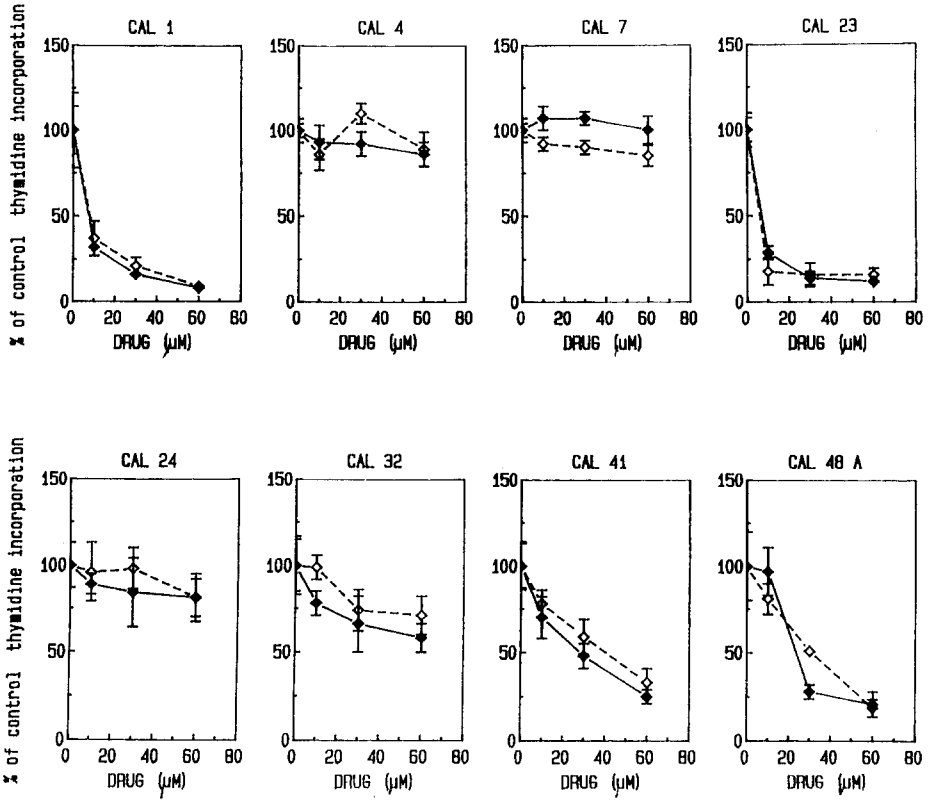

Fig. 2. Effects of nitrosoureas on [^{3}H]-thymidine incorporation by eight human melanoma cell lines (drug concentrations in μM). ◆ (*solid line*), S 10036; ◇ (*dashed line*), BCNU. Time of contact with the drugs was 150 min. The assay was carried out 78 h after drug removal (*t3*)

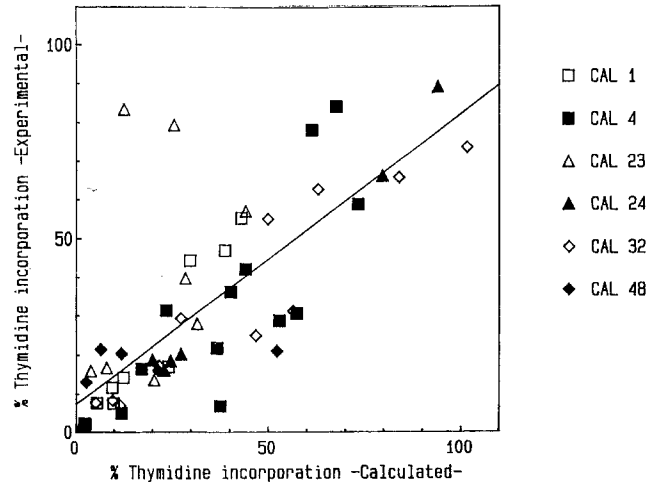

Fig. 3. Comparison of the experimental and expected effects of S 10036 and CDDP in combination on six human melanoma cell lines. *X axis*, calculated product of the individual percentage of [^{3}H]-thymidine incorporation for the two drugs acting alone, measured at t3; *Y axis*, experimental percentage of [^{3}H]-thymidine incorporation for the two drugs acting together, measured at t3 (see Table 3) for experimental conditions). Regression line according to Spearman: $y = A + r' x$; $r' = 0.821$; $n = 55$; $A = 5$; different from $y = x$; $t = 2..28$; $P < 0.03$

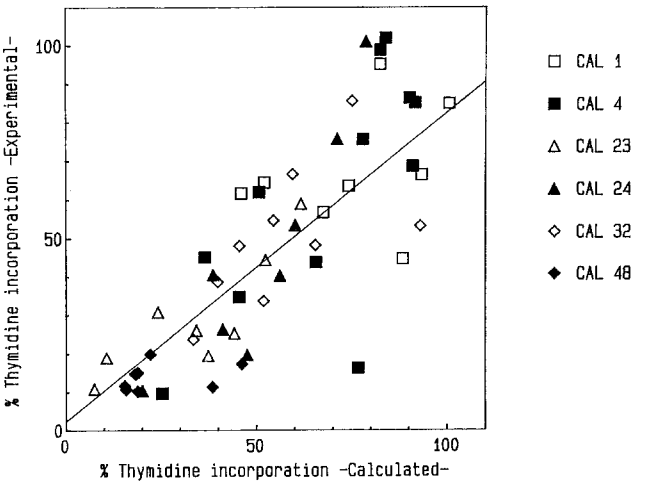

Fig. 4. Comparison of the experimental and expected effects of S 10036 and DTIC in combination on six human melanoma cell lines. *X axis*, calculated product of the individual percentage of [^{3}H]-thymidine incorporation for the two drugs acting alone, measured at t3; *Y axis*, experimental percentage of [^{3}H]-thymidine incorporation for the two drugs acting together. Regression line according to Spearman: $y = A + r' x$; $r' = 0.839$; $A = 2.4$; $n = 56$; different from $y = x$; $t = 2.17$; $P = 0.03$

Results

The respective dose-effect curves for Fotemustine (S 10036), BCNU and CCNU are presented in Fig. 1 for incubation time t3. No marked differences in cell sensitivity to the drugs were observed for any cell line and similar patterns were observed for t1 and t2. Results are summarized in Table 4. Two-thirds of the cell lines (8/12) were resistant to the drugs. The three drugs showed the same spectrum of activity.

Responses of the eight melanoma cell lines to S 10036 and BCNU are illustrated in Fig. 2 and summarized in

Table 5. Although the two drugs had the same spectrum of activity, Fotemustine induced a more marked reduction in the percentage of [^{3}H]-thymidine incorporation than did BCNU on an equimolar basis. For BCNU, there were two very sensitive, two moderately sensitive and four very resistant cell lines; for S 10036, there were two very sensitive, one sensitive, two moderately sensitive and three very resistant cell lines. There did not seem to be correlation between the proliferation index of the individual cell lines, as evaluated by thymidine incorporation in the controls and sensitivity to the three drugs, either for the 12 miscellaneous cell lines or for the 8 melanoma cell lines.

The effects of the combination of S 10036 with CDDP or DTIC on a panel of six melanoma cell lines are illustrated in Figs. 3 and 4, respectively. If the predicted percentage in reduction of [^{3}H]-thymidine incorporation is expressed for each combination as the product of the individual observed effect for each drug incubated separately, a significant correlation existed between the calculated (predicted) and the observed cytotoxic effects. Moreover, a significant difference was noted between the slope X = Y (predicted equals observed) and the slope from the data points, in favor of a synergistic effect for S 10036 + CDDP and S 10036 + DTIC.

Discussion

Fotemustine is representative of the new generation of nitrosoureas containing a special carrier group. The graft of diethyl ester phosphonic acid into the nitrosourea radical is designed to obtain better cellular penetration by the drug. Using a panel of human tumor cell lines of various origins, we demonstrated that Fotemustine and the two reference nitrosoureas BCNU and CCNU have similar spectra of activity, based on comparisons of equimolar doses. A previous phase I trial [6] suggested that Fotemustine has promising antitumor activity against melanoma, and this was confirmed by a phase II study [7]. Interestingly, in the latter study a response rate of 26.7% was shown; this value is close to the proportion of melanoma cell lines that has been found to be very sensitive to Fotemustine (2/8; 25%). Particular attention was therefore paid to the effects of this drug on a panel of eight melanoma cell lines, and the results were compared with those obtained using BCNU. A small advantage was observed for Fotemustine: 5/8 cell lines were moderately to very sensitive to this drug vs 4/8 using BCNU. This finding contrasts with a recent study [9] using another interesting grafted nitrosourea, TCNU [13], in which this drug failed to show better activity than BCNU on a panel of human small-cell lung-cancer cell lines.

The activity of Fotemustine on melanoma cell lines was further investigated by analyzing the effect of a combination of this drug with CDDP or DTIC. DTIC is the reference drug in the chemotherapeutic treatment of melanoma. CDDP has demonstrated some activity against melanoma [12] and, interestingly, has proved to be strongly synergistic with BCNU and CCNU in vitro [3]. When DTIC or CDDP were combined with Fotemustine, the observed cytostatic effects were greater than the predicted effects. This indicates that these combinations have more than additive activity on melanoma cell lines. Although there was a statistically significant difference between the slopes of simple additivity (X = Y) and those resulting from the data points, the difference was not particularly marked, and strong synergy can thus be excluded from these observations. This drug potentialization, although modest, may result from the effects of these drugs on DNA, since all three are alkylating agents. Other complementary sites can be advocated, such as their interaction with membranes [8]. In conclusion, this in vitro study supports the clinical impression that Fotemustine has interesting activity against melanoma and provides an objective means of further improving the clinical use of this drug.

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