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Evaluation of the Time-schedule Dependency for the Cytotoxic Activity of the New Vinca Alkaloid Derivative, S 12363 (Vinfosiltine)

J.L. Fischel, M. Berlion, P. Formento, J.P. Bizzari and G. Milano

S 12363 is a new vinca alkaloid derivative obtained by appending an optically active α-aminophosphonate at the C23 position of 04-deacetyl vinblastine. The present study concerns four different human tumour cell lines, which represent the spectrum of vinca alkaloid clinical activity. The influence of time exposure on S 12363 growth inhibition was studied *in vitro*. Cells were exposed to the drug during the following exposure times : 5, 15, 30 min and 1, 3, 6, 12, 24, 48, 72, 144 h. The concentrations of S 12363 applied were between 1×10^{-2} and 1×10^{3} nmol/l. The cytotoxic effects were assessed by using the methyltetrazolium (MTT) semi-automated test. Considering the IC_{50} values in terms of concentration (C) × time (T), I $(C \times T)_{50}$, it was shown that for an equal growth inhibitory effect (50% of cell death) the increased exposure times required higher cumulative drug exposures. More precisely, only very long exposure (greater than 24 h) resulted in very high I $(C \times T)_{50}$. The drug exposure ratios which correspond to I $(C \times T)_{50}$ values for 144 h divided by the I $(C \times T)_{50}$ values for 0.25 h ranged between 2.8 and 18.3. If T and C had symmetrical effects on the final growth inhibition, the I $(C \times T)_{50}$ ratios should have been equal to one. For all cell lines investigated there were similar dose–response curves following two types of S12363 exposure: a single day exposure or three successive daily exposures, the total C × T values being the same in both experimental situations. The basic pharmacological information provided by the present study may encourage further clinical trials of this potentially interesting new vinca alkaloid.
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INTRODUCTION

S 12363 (VINFOSILTINE) IS A new vinca alkaloid derivative obtained by appending an optically active α-aminophosphonate at the C23 position of 04-deacetyl vinblastine. This compound is very promising, since it is 72 and 36 times more cytotoxic than vincristine and vinblastine, respectively, when tested on a panel of murine and human tumour cell lines using the methyltetrazolium (MTT) assay [1]. It is the objective of preclinical investigations for a new anticancer drug to supply rational guidelines which may be useful in providing the basis for future clinical trials [2]. More precisely, these guidelines could outline suggestions for an optimal administration schedule based on

Table 1. *Human tumour cell lines investigated*

Cell line	Tumour type	Obtained from	Doubling time (h)	Initial density (cells/well)
MCF7	Breast	Prof. H. Rochefort Inserm, Montpellier, France	41	2500
SKOV3	Ovary	American Type Culture Collection (ATCC) Rockville, U.S.A. Ref HTB 77	38	3500
WIDR	Colon	EORTC, Clonogenic Assay Screening Study Group (CASSG)	41	3000
SRO	Lung (epidermoid)	Prof. J. Juillard UCLA, Los Angeles U.S.A.	43	6000

information concerning the time schedule dependency of the drug's cytotoxic effects. Antimetabolites, such as 5-fluorouracil and cytarabine, constitute good examples in which the advantages of continuous infusion scheduling were recognised both at the bench [3] and during clinical practice [4]. Although the compound S 12363 shares common structural properties with other classical vinca alkaloids, its grafted aminophosphonate radical can confer quite specific cellular pharmacological behaviour concerning, for example, a modified cellular membrane transport. Thus, it may not be valid to extrapolate to S 12363 the experimental knowledge on time schedule dependency of other vinca alkaloids [5].

The present study concerns four different human tumour cell lines, which are representative of the spectrum of vinca alkaloids clinical activity. The influence of time exposure on S 12363 growth inhibition was studied in depth under experimental conditions where the key parameter [time(T)–concentration(C) product] [6] was taken into consideration. In addition, the difference in growth inhibitory effects generated by single and sequential exposure of cells to S 12363 was analysed.

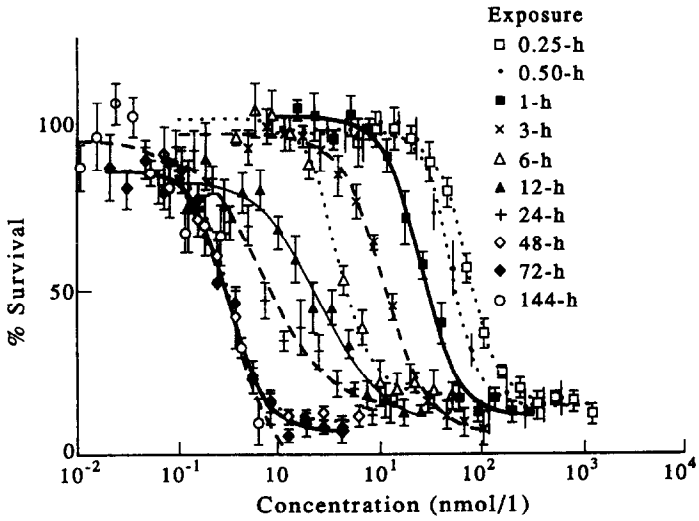

Fig. 1. Evolution of MCF7 cell survival as a function of S 12363 concentration. Each experimental point was performed six times. Vertical bars indicate S.D.

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MATERIALS AND METHODS

Chemicals

S 12363 was obtained as a pure powder from Servier laboratories (Courbevoie, France). Working solutions were prepared extemporaneously by dilution in the culture medium. Dulbecco's modified Eagle's medium (DMEM), L-glutamine and fetal bovine serum (FBS) were from Gibco (Paisley, U.K.). Penicillin and streptomycin were from Merieux (Lyons, France). Transferrin was from Flow Laboratories (Irvine, U.K.). The MTT test was performed with 3-(4-5 dimethylthiazol-2-yl)- 2,5 diphenyltetrazolium bromide (MTT) and dimethylsulphoxide (DMSO). As already shown for classical vinca alkaloids [7], S 12363 is stable in solution for several weeks.

Experimental conditions

The four human tumour cell lines used are described in Table 1. Cell lines were free of mycoplasma contamination. Cells were routinely cultured in a humidified incubator (Sanyo) at 37°C with an atmosphere containing 8% CO_2 in air. Cell lines were grown in DMEM medium supplemented with 10% FBS, penicillin (50 000 U/l), streptomycin (80 μ mol/l) and L-glutamine (2 mmol/l). In brief, cells were grown in 96-well microtitre plates in their respective culture medium; 24 h after subculture they were exposed to the drug for the following durations : 5, 15, 30 min and 1, 3, 6, 12, 24, 48, 72, 144 h. The concentrations of S 12363 applied were between 1×10^{-2} and 1×10^{3} nmol/l. These concentrations were selected according to the range of IC_{50} values previously reported [1]. Other supplementary experiments concerned the comparison between single- and 3-day exposure of cells to S 12363 : the duration of exposure was 1 h and the concentrations tested during the 3-day exposure were one third of those tested during single exposure; thus the total C × T was the same in both conditions.

Evaluation of growth inhibition

The growth inhibitory effects were assessed by using the MTT semi-automated test [8] after 6–8 days of exposure in the 96-well incubating plates. The MTT incubation time was 4 h. Results were expressed as the relative percentage of absorbance compared to controls without drugs. Absorbance was measured at 540 nm (Titertek Twinreader). Each dose point was performed six times. All experiments were duplicated. IC_{50} was defined

Table 2. Evolution of IC_{50} and I $(C \times T)_{50}$ values as a function of the exposure time

Cell lines			Duration of exposure (h) 0.25	0.50	1	3	6	12	24	48	72	144	Drug exposure ratio
SRO	A	C	27.9	16.8	13.6	5.4	2.3	1.8	0.82	0.92	0.95	0.89	
		C × T	7.0	8.5	13.6	16.2	13.8	21.6	19.6	44.1	68.4	128.1	18.3
	B	C	49.0	34.6	14.8	4.9	2.9	1.9	1.1	0.72	0.73	0.66	
		C × T	12.2	17.3	14.8	14.7	17.4	22.8	26.4	34.5	52.5	95.0	7.8
SKOV3	A	C	68.4	65.2	32.3	15.9	10.7	5.2	2.7	1.45	1.06	0.93	
		C × T	17.1	32.6	32.3	47.7	64.2	62.4	64.8	69.6	76.3	134.0	7.8
	B	C	113.9	64.2	34.6	13.9	8.6	6.4	3.9	0.52	0.50	0.56	
		C × T	28.5	32.1	34.6	41.7	51.6	76.8	93.6	25.0	36	80.6	2.8
MCF7	A	C	58.6	53.2	28.7	11.5	7.6	3.4	0.79	0.68	0.43	0.54	
		C × T	14.6	26.6	28.7	34.5	45.6	40.8	18.6	32.6	30.9	77.8	5.3
	B	C	72.1	50.1	25.5	10.9	3.9	2.6	0.72	0.29	0.32	0.33	
		C × T	18.0	25.1	25.5	32.7	23.4	31.2	17.2	13.9	23.0	47.5	2.6
WIDR	A	C	54.0	26.1	14.9	5.9	3.6	1.9	0.72	0.57	0.57	0.62	
		C × T	13.5	13.0	14.9	17.7	21.6	22.8	17.2	27.4	41.0	89.3	6.6
	B	C	74.5	38.2	20.5	7.1	3.9	3.3	0.55	0.59	0.44	0.42	
		C × T	18.6	19.3	20.5	21.3	23.4	39.6	13.2	18.7	31.6	60.4	3.2

A and B refer to separate experiments. C = IC_{50} value (nmol/l); C × T = the product of IC_{50} × exposure time (nmol/l × h). Drug exposure ratio = the ratio of C × T (144 h)/C × T (0.25 h).

as the drug concentration causing a 50% reduction in growth compared to controls. I $(C \times T)_{50}$ was defined as the drug exposure (C × T, product of the applied concentration by the duration of exposure) causing 50% reduction in growth compared to controls. IC_{50} and I$(C \times T)_{50}$ values were automatically computerised from the sigmoid curves generated from the dose–response graphs ("Graph Pad", ISI Software).

RESULTS

Figure 1 illustrates the evolution of the growth inhibition according to the applied S 12363 concentration for the MCF7 cell line and for different durations of cell exposure to the drug. For all cell lines investigated there were steep dose–response curves; it can be noted that the sigmoid curves were almost superimposed for the highest durations of exposure : 48, 72 and 144 h. Figure 2 represents the evolution of cytotoxicity as a function of the total drug exposure (C × T) for the SKOV3 cell line; it is noteworthy that for any given C × T value, the lowest growth inhibitory effects correspond to the greatest exposure time. Results obtained for all cell lines and all experiments are summarised in Table 2. The various cell lines exhibited some differences in S 12363 sensitivity with SRO cells being the most sensitive, MCF7 cells and WIDR cells showing an intermediary sensitivity and SKOV3 cells being relatively resistant. Considering the IC_{50} values in terms of C × T, it was shown that for an equal growth inhibitory effect (50% of cell death) the increased exposure times necessitated highest drug exposures. The drug exposure ratios which correspond to I $(C \times T)_{50}$ values for 144 h divided by the I $(C \times T)_{50}$ values for 0.25 h ranged between 2.8 (SKOV3, second experiment) and 18.3 (SRO, first experiment). If time and concentration had symmetrical effects on the final growth inhibition, the I $(C \times T)_{50}$ ratios should have been equal to one. Figure 3 illustrates the overall relationship between I $(C \times T)_{50}$ units and the exposure time: there was a linear, positive and significant correlation between these two variables ($r = 0.63$, $P = 0.001$) for MCF 7; ($r = 0.69$, $P = 0.004$) SKOV3; ($r = 0.88$, $P < 0.0001$) WIDR and ($r = 0.98$, $P < 0.0001$) SRO cells. Apart from this general relationship between I $(C \times T)_{50}$ and the duration of exposure, a close stepwise analysis of the evolution of the data shows that 24 h is the time where an inflexion occurs in the increasing I $(C \times T)_{50}$ values during time increments; this was particularly marked for WIDR and MCF 7 cell lines.

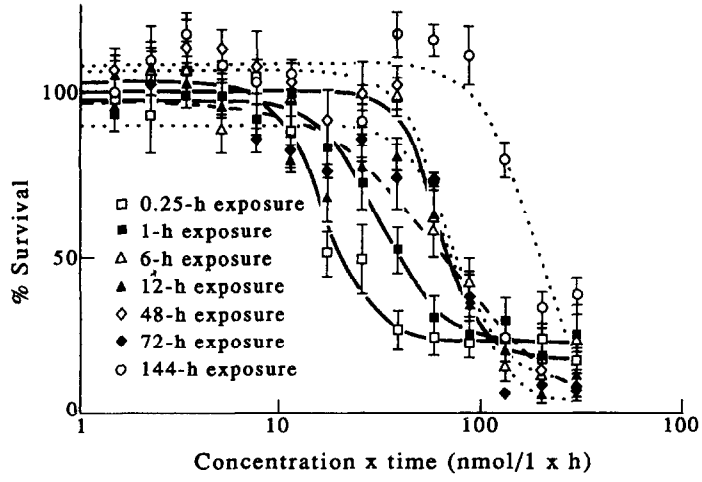

Fig. 2. Evolution of SKOV3 cell survival as a function of S 12363 (C × T). Each experimental point was performed six times. Vertical bars indicate S.D.

Figure 4 shows that for all cell lines investigated there were similar dose–response curves following two types of S 12363 exposure: a single day exposure or three successive daily exposures, the total C × T values being the same in both experimental situations.

DISCUSSION

The present data indicate that the growth inhibitory effects generated by the new vinca alkaloid S 12363 are essentially

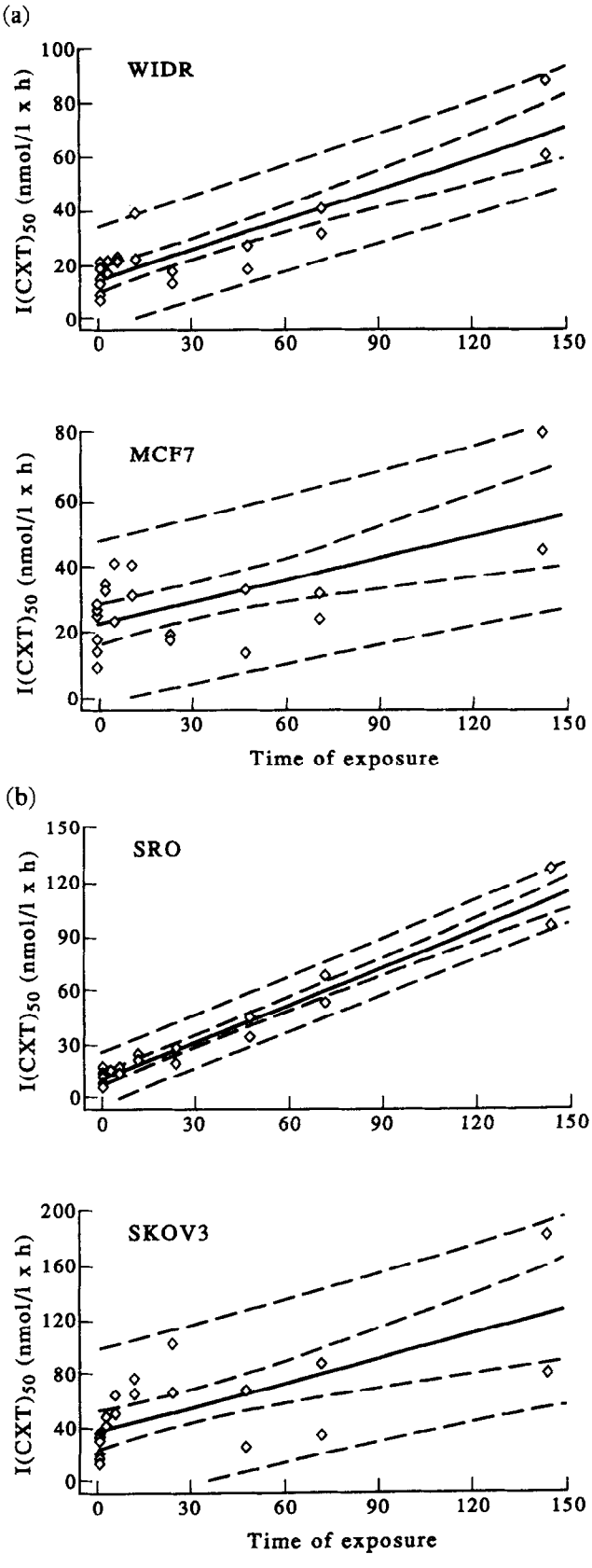

Fig. 3. Evolution of the I $(C \times T)_{50}$ values as a function of the duration of drug exposure. (a) WIDR and MCF7 cells lines; (b) SRO and SKOV3 cell lines. Linear correlations : $r = 0.88$, $P < 0.0001$; $r = 0.63$, $P = 0.001$; $r = 0.98$, $P < 0.0001$; $r = 0.69$, $P = 0.0004$ for WIDR, MCF7, SRO and SKOV3, respectively.

dependent on the applied concentration and that the duration of exposure is a lesser determining factor for S 12363 antitumour activity. This was observed for all four human tumour cell lines investigated and for two separate experiments. The antineoplastic activity of vinca alkaloids is attributed to their ability to bind to cellular microtubules and inhibit cell division. The cytotoxic effect of vinca alkaloids is expressed in M-phase with drug binding to tubulin being maximal in S-phase. This makes these drugs relatively cell-cycle specific. Precisely for cell-cycle specific drugs, such as antimetabolites, the antitumour effect is preferentially linked to the total drug exposure ($C \times T$), thus taking into account not only the concentration itself but also the duration of contact between cellular targets and the drug, this allowing a maximum of cells to enter in the drug sensitive phase [8].

Apart from the compound S 12363, the classical vinca alkaloids generally demonstrate increased growth inhibitory activity with prolonged exposure; experimental data have been extensively reviewed by Ratain *et al.* [9] where it appears that prolonged exposure time-dependent cytotoxic effects may vary with the vinca alkaloid considered and thus no definitive general rule can be drawn on this aspect. Ludwig *et al.* [10] with experimental conditions comparable to those presently used and comparing the *in vitro* cytotoxicity of a 200-h vinblastine exposure to the standard 1-h exposure in 77 tumour specimens (clonogenic assay) found that the IC_{50} ratio (IC_{50} for 1-h exposure divided by IC_{50} for 200-h exposure) was greater than 200 in most tumours thus suggesting an advantage for prolonged exposure. Matsushima *et al.* [11] comparing 24-h and 1-h exposure to vinblastine and vindesine to lung adenocarcinoma cells, found an IC_{50} ratio for vindesine and vinblastine of 36 and 18, respectively, thus indicating an advantage for prolonged exposure only for vindesine. An explanation for these differences between time-scheduled dependencies of classical vinca alkaloids and S12363 may lie in their respective cellular pharmacology. For instance, significant differences in drug efflux have been found between vinblastine and vindesine [12]. Vinca alkaloids are considered to enter cells by energy-dependent transport mechanisms [8]. The grafting of amino acid derivatives onto vinblastine has been performed in order to facilitate and improve transport and led to molecules with interesting pharmacological properties such as vinglycinate [13] and vintryptol [14]. This stimulated the synthesis of a series of α-aminophosphonate derivatives of vinblastine [15], the most active compound being S 12363 [16]. S12363 is on average 36–72 times more cytotoxic than vinblastine and vincristine, respectively, for human tumour cell lines [1, 17, 18]. Although there are not yet strict experimental data to support it, it can be hypothesised that one of the reasons for explaining the concentration rather than time dependency of S 12363 growth inhibitory effects may be a facilitated cell uptake. Early clinical trials of S 12363 have been undertaken [19–21]. Limiting toxicity is haematological. It is quite conceivable that various schedules of drug administration could be considered in the future use of this drug. For continuous infusions, the present results suggest that prolonging time exposure over 24 h is not advisable since there were no further growth inhibitory benefits gained with exposure times up to 144 h (Fig. 1, Table 2). On the other hand, comparing single to 3-day dose, similar growth inhibitory effects were found thus suggesting that single dose and intermittent schedules may be equivalent for antitumour effects. To conclude, keeping in mind the unavoidable oversimplification of *in vitro* models as compared to the whole living organism, the basic pharmacological information brought by the present study may

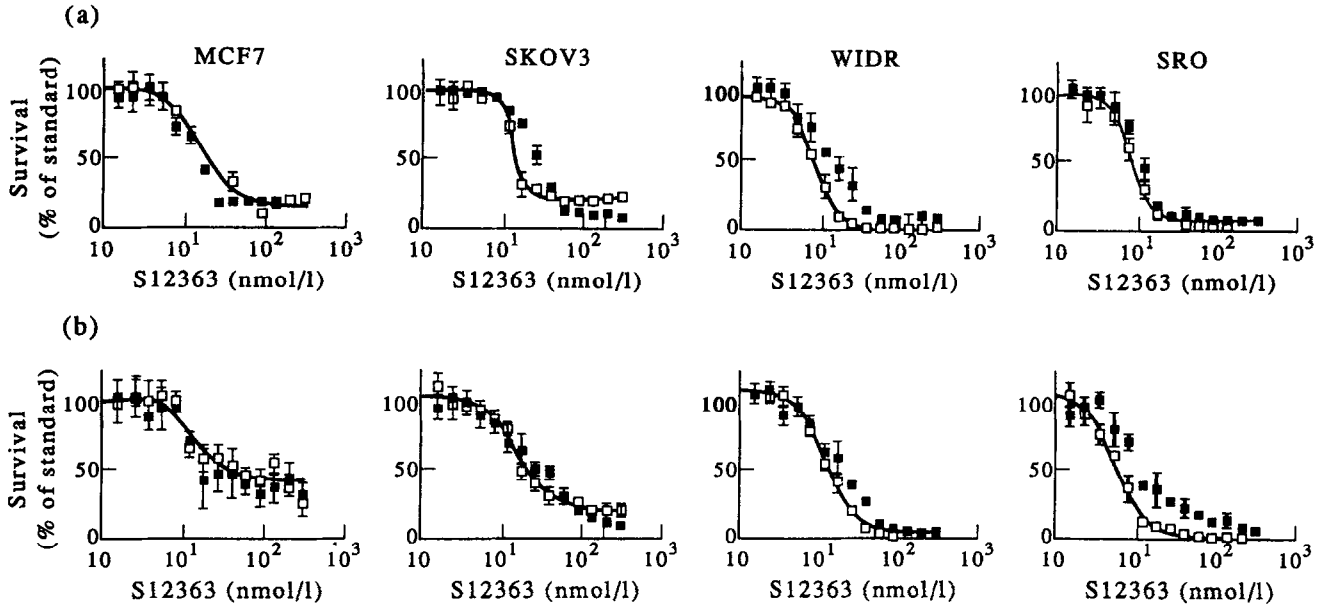

Fig. 4. Evolution of cell survival as a function of S 12363 concentration. Open square : single day exposure (1 h, full dose); filled square : 3-day exposure (1 h and full dose/3 each day). The total drug exposure being the same, the curves are thus represented with an identical concentration scale. Each experimental point was performed six times. Vertical bars indicate S.D. (a) First experiment; (b) second experiment.

be useful as an argument for setting up further clinical trials of this potentially interesting new vinca alkaloid.

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