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Relationship between the cellular accumulation and the cytotoxicity of S12363, a new vinca alkaloid derivative

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Summary. S12363, a new vinca alkaloid derivative, was considerably more cytotoxic to murine L1210 cells and five human tumor cell lines (HL60, HT-29, COLO 320DM, NCI-H460, and PANC-1) than was vincristine (VCR) or vinblastine (VLB). S 12363 bound to tubulin in crude extracts from brain or L1210 cells with an affinity similar to that of VLB and VCR (apparent K_d value: 1.1–1.6, 1.2–1.7, and 0.6–0.8 μM, respectively). After 1 h exposure, the accumulation of 20 nM [^{3}H]-S 12363 by L1210 cells was 4- to 18-fold that of [^{3}H]-VLB and [^{3}H]-VCR, respectively. After the cells had been preloaded for 1 h with the labeled drugs and then incubated for 3 h in drug-free medium, 37%–55% of the [^{3}H]-S 12363 was retained by the cells vs 36%–47% of the [^{3}H]-VCR and <6% of the [^{3}H]-VLB. Similar results were obtained for the five human cell lines tested. The accumulation factors (intracellular vs extracellular concentrations) found for [^{3}H]-S 12363 (54- to 167-fold) were significantly higher than those observed for [^{3}H]-VCR (5- to 14-fold) or [^{3}H]-VLB (19- to 41-fold). >90% of the radioactivity extracted from L1210 cells that had been treated with [^{3}H]-S 12363 was recovered as unmodified drug, demonstrating that [^{3}H]-S 12363 was not metabolized by these cells. S 12362, which differs from S 12363 only in the absolute configuration of the asymmetric carbon atom of its α-aminophosphonic side chain, was 300 times less cytotoxic, bound to tubulin with a lower affinity (apparent K_d value, 4.9–9.6 μM), and was neither accumulated nor retained by the cells. Taken together, these results demonstrate that the potency of S 12363 is due at least in part to its cellular accumulation and retention.

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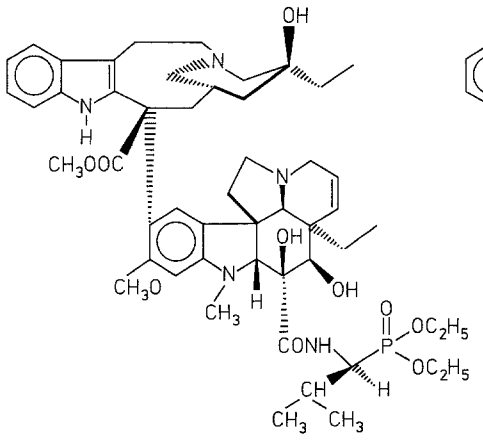
Introduction

S 12363 is a new vinca alkaloid derivative, synthesized by grafting an optically active α-aminophosphonate on *O*4-deacetyl vinblastine [17]. This compound has been shown to be highly cytotoxic to human tumor cell lines in vitro [22, 24], more active, and 20 times more potent in vivo than vinblastine (VLB) or vincristine (VCR) in both murine transplantable tumors and human tumor cell lines xenografted into nude mice [16, 24]. Its epimer, S 12362, which differs only in the absolute configuration of the asymmetric carbon atom of its side chain, is 300 times less cytotoxic and 1,000 times less potent in vivo [24]. The latter compound is thus an interesting tool for the study of the mechanism of action of S 12363.

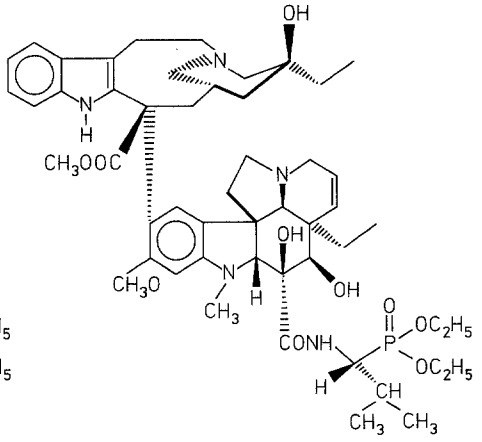
The cytotoxic and antitumor properties of vinca alkaloids may be modulated by differences in their uptake and cellular retention rather than by discrepancies in their affinity to their main target, the tubulin dimer. The different growth inhibition induced by VCR and VLB has been found to correlate with their intracellular retention by tumor cells both in vitro [6–8, 18, 25] and in vivo [11, 12]. This differential retention of VCR and VLB and, hence, their selective toxicity, has been correlated with the stability of the drug-tubulin complex as measured in cytosol and in tumors in vivo [4, 13, 14].

The basis for the high potency of S 12363 was not clearly understood at this time, since we have shown that this compound is as efficient as VCR and VLB in inhibiting brain-tubulin polymerization in vitro [23]. Moreover, the high and rapid blockade in mitosis of L1210 cells that have been treated with S 12363 suggested a rapid uptake of this compound by the cells [19].

The aim of the present study was to determine whether the potency of S 12363 is attributable to its high accumulation by tumor cells. Since tumor-cell tubulin could differ from brain tubulin in its susceptibility to vinca alkaloid derivatives, we compared the affinity of S 12363 for brain vs L1210-cell tubulin. The accumulation and retention of [^{3}H]-S 12363 by tumor cells in culture (L1210 and five human cell lines) was compared with those of its inactive



S 12363



S 12362

Fig. 1. Structure of S 12363 and S 12362

epimer, [^{3}H]-S 12362, and two standard vinca alkaloids, [^{3}H]-VCR and [^{3}H]-VLB.

Materials and methods

Drugs. Vincristine sulfate was purchased from Roger Bellon, France, and vinblastine sulfate was obtained from Eli-Lilly, France; both drugs were dissolved in water prior to their use. S 12363 is (1S)-1-[3-*O*4-deacetyl-3-demethoxy-carbonylvinca-leukoblastinyl)-carbonylamino]-2methylpropyl-phosphonic acid diethylester sulfate and S 12362 is the 1R isomer (Fig. 1). S 12362 and S 12363 were synthesized as described elsewhere [17] and were dissolved in ethanol and diluted with complete culture medium. They were purified by high-performance liquid chromatography (HPLC) and were found to be approx. 99% pure. [^{3}H]-VCR, [^{3}H]-VLB, [^{3}H]-S 12362, and [^{3}H]-S 12363 (5–20 Ci/mmol) were purchased from Amersham, were found to be 95%–98% pure, and were shown to produce little tritium exchange with water under the conditions of these experiments. [^{3}H]-S 12362 and [^{3}H]-S 12363 were used within the first 3 weeks; they were controlled and repurified by Amersham.

Cell culture and cytotoxicity assay. All cell lines were obtained from the American Type Culture Collection (Rockville, Md., USA) except for NCI-H125 and NCI-H460, which were provided by the DCT Tumor Repository (NCI, Frederick, Md., USA). They were cultivated in RPMI 1640 medium supplemented with 10% decomplemented fetal calf serum, 2 mM L-glutamine, 50 IU penicillin/ml, 50 µg streptomycin/ml, and 10 mM HEPES buffer (pH 7.4). The cell volume was determined using a model ZM Coulter counter (Coultronics) fitted with a 100-µm aperture tube. The particle-size analyzer (Coulter Channelyzer C1000) was interfaced with a Vax minicomputer (Digital Equipment) and was calibrated with polystyrene microspheres measuring 8.4 µm in diameter. The cell volume was calculated and used to estimate the total intracellular concentration of drug.

The microculture tetrazolium assay was performed as previously described [1, 24]. Cells were exposed to the drugs (nine concentrations in triplicate) for 1 h, washed, and then incubated in drug-free medium for the temporal equivalent of four cell divisions. The experimental conditions were chosen so as to maintain cells in the exponential phase of growth and to obtain a linear relationship between the optical density and the number of cells. The results were expressed as IC_{50} values (the drug concentration required to reduce the optical density of treated cells by 50% in relation to untreated control values).

Binding to tubulin. The binding of tritiated compounds to a tubulin-enriched protein fraction was measured in a 100,000 *g* supernatant (S100) prepared from a dog brain [26] or from L1210 cells. Briefly, the brain was homogenized in 0.1 M MES buffer, 1 mM ethylene glycol tetraacetic acid (EGTA), 0.5 mM $MgCl_2$, 4 M glycerol and 0.1 mM guanosine triphosphate (GTP); pH 6.5 at 0° C. The homogenate was centrifuged at 100,000 *g* for 80 min at 4° C and the supernatant (S100) was stored at –80° C. The protein content was 15 mg/ml as measured using the Lowry procedure [20]. For the preparation of L1210 S100, 20 l culture ($1-2\times10^6$ cells/ml) were centrifuged and washed with phosphate-buffered saline (PBS). The cell pellet was resuspended in NGM buffer (10 mM phosphate buffer, 0.1 mM GTP, 1 mM $MgCl_2$; pH 6.8) and incubated for 15 min at 0° C. The cell suspension was homogenized and centrifuged as described above. The S100 (6 mg protein/ml) was stored at –80° C. Binding was measured in a final volume of 1 ml containing 0.9–1 mg brain S100 proteins or 2 mg L1210 S100 proteins and 1 µM [^{3}H]-VLB (0.125 µCi) in NGM buffer.

The reaction mixture was incubated for 15 min at room temperature and filtered under gravity on a DE 81 filter (Whatman) that had been equilibrated with NM buffer (10 mM sodium phospate buffer, 1 mM $MgCl_2$; pH 6.8) for 3 h [30]. After 2 min, the filters were rapidly (<40 s) washed four times with 5 ml ice-cold NM buffer and the radioactivity was counted. Nonspecific binding (about 10% of total binding) was measured in the presence of 100 µM VLB and was subtracted from each value. The drugs were tested at concentrations of 0.6–10 µM, and the apparent dissociation constant (K_d) was calculated according to Cheng and Prusof [5].

Accumulation and retention of tritiated compounds by cells in culture. Adherent cells in the exponential phase of growth were harvested with 0.05% trypsin, rinsed, and incubated for 2 h at 3×10^5 cells/ml. Nonadherent cells in the exponential phase of growth were used at the same density. Cells were incubated with the tritiated compounds exactly under the conditions used for the cytotoxicity assay (i.e., in complete culture medium at 37° C). The cells were exposed to the indicated concentrations of labeled drugs ([^{3}H]-VCR, [^{3}H]-VLB, [^{3}H]-S 12363: 0.02–2 µCi/ml; [^{3}H]-S 12362: 1–16 µCi/ml).

The accumulation of drugs was measured as described elsewhere [6], with slight modifications. Two aliquots of 1 ml were collected at various times and centrifuged for 20 s at 12,000 *g*, the cell pellet was washed twice with cold 0.15 M NaCl and then lysed with 100 µl 1% Triton X-100 for 1 h, and the radioactivity was counted. The values obtained after 5 s incubation at 0° C, which were probably attributable to the nonspecific adsorption of drugs on the cell surface, were subtracted from each value. These values represented 10%–14% ([^{3}H]-VCR, [^{3}H]-VLB, [^{3}H]-S 12363) and 40% ([^{3}H]-S 12362) of the uptake by cells after 1 h incubation at 37° C.

For the measurement of drug retention, cells were incubated for 1 h at 37° C with different concentrations of labeled drugs, washed, and resuspended in complete culture medium. At various times, two aliquots of 1 ml were collected and centrifuged, the cell pellet was washed with 1 ml cold 0.15 M NaCl, and the radioactivity was counted. Control incubations in the absence of cells were processed in parallel and the corresponding values were subtracted. These background values repre-

Table 1. Inhibition of [^{3}H]-VLB binding to tubulin

	Apparent K_d (μM)	
	Brain S100	L1210 S100
VCR	0.60±0.19	0.85±0.21
VLB	1.18±0.26	1.72±0.55
S 12362	4.89±0.77	9.62±5.20
S 12363	1.09±0.13	1.63±0.20

The binding of 1μM [^{3}H]-VLB to brain or L1210 tubulin of crude extracts was measured in the absence or presence of increasing concentrations (0.6–10 μM) of the unlabelled competitors. The apparent K_d values were calculated according to Cheng and Prusof [5]. Data represent mean values ± SEM ($n \geqq 3$)

sented <10% of the [^{3}H]-VCR and [^{3}H]-VLB and 10%–15% of the [^{3}H]-S 12362 and [^{3}H]-S 12363.

HPLC analysis. L1210 cells (40 ml at 5×10^5 cells/ml) were incubated for 3 h with 50 nM [^{3}H]-S 12363 (1 μCi/ml). Cells were centrifuged, washed twice with cold PBS, and transferred to another tube, and the radioactivity in the cell pellet was extracted with 1 ml ethanol for 1 h at 4° C. Unlabeled S 12363 (0.1 mM) was then added and the suspension was centrifuged. The supernatant was filtered through a 0.45 μm Millipore HV filter and concentrated to a volume of 100 μl, 10 μl of which was injected into the HPLC system equipped with a Lichrosorb RP 18 column (Merck). The radioactivity was isocratically eluted with a 10-mM Na_2HPO_4/methanol (70:30, v/v) solution. Fractions of 1 ml were collected and the radioactivity was measured.

Results

Binding to tubulin

The interaction of the derivatives with the brain-tubulin fraction and the L1210 tubulin-enriched protein fraction was studied by competition with [^{3}H]-VLB. The binding of 1 μM [^{3}H]-VLB was linear at concentrations of up to 1 mg/ml brain S100 proteins or 2 mg/ml L1210 S100 proteins, reached a plateau after 5 min, and remained stable for at least 2 h (data not shown). The binding of 1 μM [^{3}H]-VLB was then displaced by increasing the concentrations of the competitors. The apparent K_d values calculated from the IC_{50} values according to Cheng and Prusof [5] are listed in Table 1. These K_d values showed that the binding affinity of VCR, VLB, and S 12363 was similar, whereas S 12362 was about 5 times less potent than S 12363. Similar values were obtained using tubulin from brain or L1210 cells (Table 1).

Cytotoxicity of the compounds to cells in culture

The IC_{50} values obtained after 1 h contact are listed in Table 2. S 12363 was highly cytotoxic to all cell lines except the COLO 320DM line, which showed the cross-resistance pattern characteristic of multidrug resistance (data not shown). On average, the potency of S 12363 was 180-fold that of VCR, 40-fold that of VLB, and 300-fold that of S 12362.

Accumulation and retention by L1210 cells in culture

To determine whether the higher potency of S 12363 was attributable to better accumulation as compared with the other drugs, the accumulation of the tritiated compounds was measured at pharmacologically relevant concentrations (including their IC_{50}) under the same experimental conditions used for the cytotoxicity assay. Figure 2 shows the accumulation and retention of the four tritiated compounds by L1210 cells as a function of time and at increasing concentrations in the culture medium.

As previously reported [6–8, 18, 25], [^{3}H]-VLB was rapidly taken up by the cells, with a plateau being obtained within 15–30 min. In contrast, the rate of penetration of [^{3}H]-VCR was slower, with no plateau being evident after 3 h incubation. At the same concentrations (20–400 nM) and after 1 h incubation, the accumulation of [^{3}H]-VLB into cells was about 3-fold that of [^{3}H]-VCR. The uptake of [^{3}H]-S 12363 was measured at low concentrations of 5–40 nM, including its IC_{50}. The rate of penetration was rapid, and no plateau was evident after 3 h. A high accumulation of [^{3}H]-S 12363 by cells was observed, since 40 nM [^{3}H]-S 12363 and 200 nM [^{3}H]-VLB led to similar intracellular concentrations (Fig. 2). The uptake of [^{3}H]-S 12362, first measured at the same low concentrations, was hardly detectable; therefore, we used higher concentrations (400 and 1,000 nM) as shown in Fig. 2. The uptake

Table 2. Cytotoxicity of the vinca alkaloid derivatives

	IC_{50} (nM)			
Cell lines	VCR	VLB	S 12362	S 12363
Murine:				
L1210 (leukemia)	121.3± 40.4	180.5± 31.8	4,342.5±356.5	14.4± 1.3
Human:				
HL60 (leukemia)	27.1± 3.7	50.7± 12.2		1.9± 0.7
HT-29 (colon)	214.5± 20.5	215.3± 58.8		6.5± 1.5
COLO 320DM (colon)	56,552.0±15841.5	3,577.3±673.3		361.7±78.1
NCI-H460 (lung)	1,057.1± 378.5	340.4±107.5		4.6± 1.0
PANC-1 (pancreas)	5,939.7± 853.9	532.0±111.4		10.7± 1.3

Cells were incubated with the drugs for 1 h, washed, and then incubated in drug-free medium
Data represent mean values ± SEM ($n \geqq 3$) as determined using the microculture tetrazolium assay

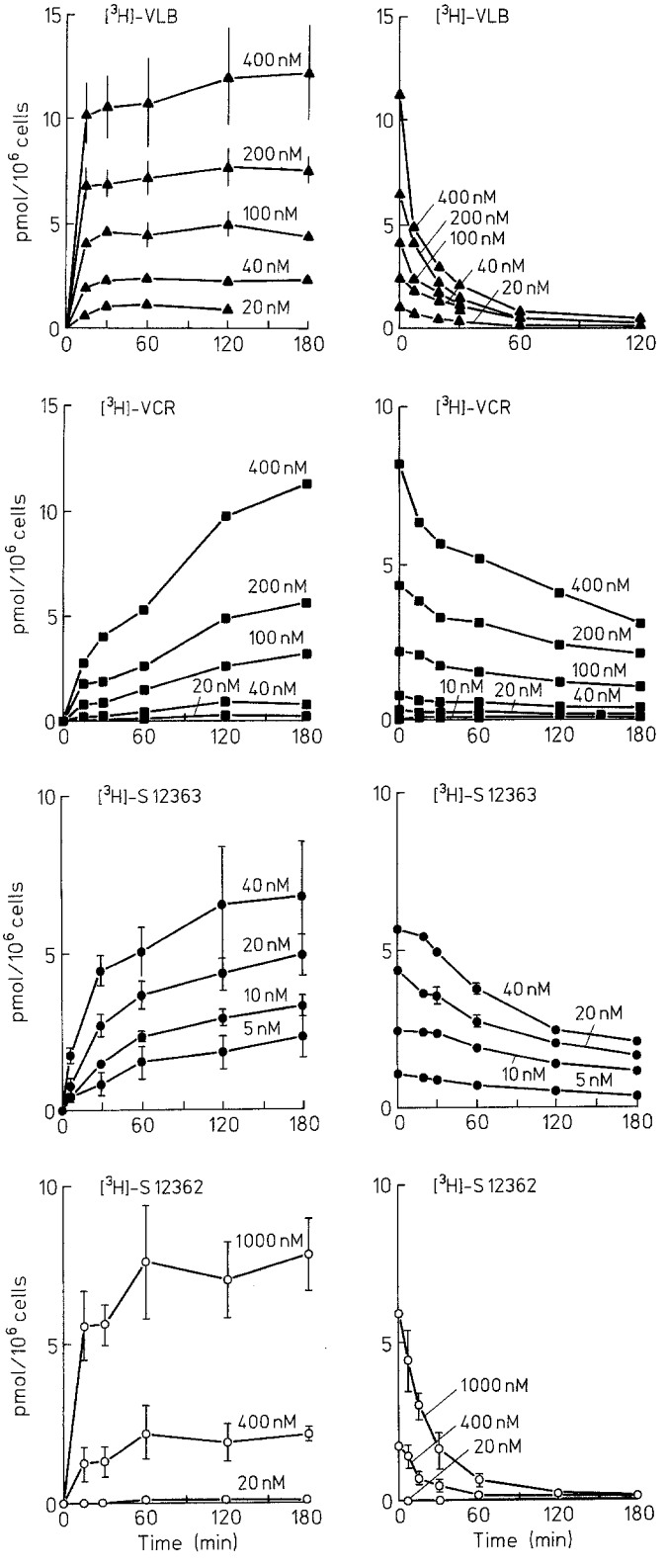


Fig. 2. Uptake and retention of the tritiated vinca alkaloid derivatives by L1210 cells. For measurements of uptake (*left panels*), cells were incubated with the indicated concentrations of labeled drugs, and the intracellular radioactivity was measured at the indicated times and expressed as pmol labeled drug/10^6 cells. For determinations of retention (*right panels*), cells were preloaded for 1 h under the conditions described above, washed, and then incubated in drug-free culture medium. Cell-associated radioactivity was measured as indicated above. Points represent the mean of 2 ([3H]-VCR) or 3 values ± SEM; bars indicate that the values are larger than the symbols

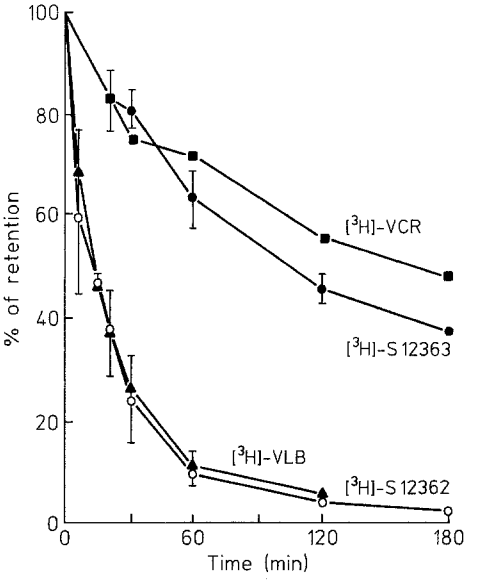


Fig. 3. Comparative cellular retention of the tritiated vinca alkaloid derivatives by L1210 cells. The cells were loaded for 1 h with 200 nM [3H]-VCR (■——■), 100 nM [3H]-VLB (▲——▲), 20 nM [3H]-S 12363 (●——●), or 1,000 nM [3H]-S 12362 (○——○) and were then washed and incubated in drug-free medium. The cell-associated radioactivity was measured at the indicated times and expressed as a percentage of the initial value. Retention values of 100% were equivalent to 4.3 ([3H]-VCR), 4.4±0.6 ([3H]-VLB), 4.5±0.4 ([3H]-S 12363), and 6.2±0.7 pmol/10^6 cells ([3H]-S 12362)

plateaued after 1 h incubation and the cellular accumulation was very low, considering the high concentrations used. After 1 h incubation of the cells with drug concentrations of 20 nM, the intracellular concentration of [3H]-S 12363 was significantly higher than that of [3H]-VLB, [3H]-VCR, and [3H]-S 12362 (4, 18, and 40 times higher, respectively).

The retention of the labeled compounds was measured in L1210 cells that had been preloaded for 1 h with increasing concentrations of the compounds, washed, and transferred to drug-free medium. In agreement with previously published results [6, 7, 18], we found that the efflux of [3H]-VLB was rapid, whereas [3H]-VCR was retained longer by the cells (Fig. 2). An important difference was observed between the two epimers: the efflux of [3H]-S 12363 was low, being similar to that of [3H]-VCR, whereas 90% of the [3H]-S 12362 was released from the cells after 1 h. To obtain a better comparison of the rates of efflux, we preloaded the cells for 1 h with concentrations of the drugs that resulted in almost identical intracellular concentrations (4–5 pmol/10^6 cells).

Figure 3 shows the kinetics of drug retention expressed as a percentage of the initial radioactivity. [3H]-VCR and [3H]-S 12363 exhibited a similarly slow rate of efflux, whereas [3H]-VLB and [3H]-S 12362 were rapidly released from the cells. After 3 h incubation in drug-free medium, 48% of the [3H]-VCR and 37% of the [3H]-S 12363 remained associated with the cells vs <6% of the [3H]-VLB and 2% of the [3H]-S 12362.

We then studied two properties of the [3H]-S 12363 accumulation by L1210 cells, namely, saturability and sen-

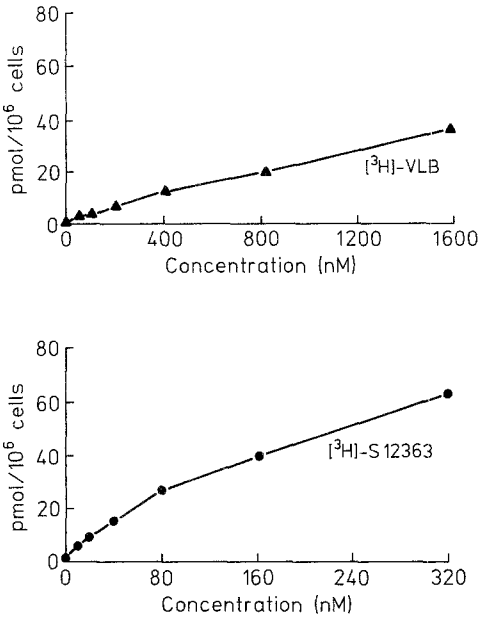


Fig. 4. Cellular accumulation of [^{3}H]-VLB (*upper panel*) and [^{3}H]-S 12363 (*lower panel*) by L1210 cells as a function of concentration. The cellular accumulation of the labeled drugs was measured after 1 h incubation of L1210 cells at the indicated concentrations

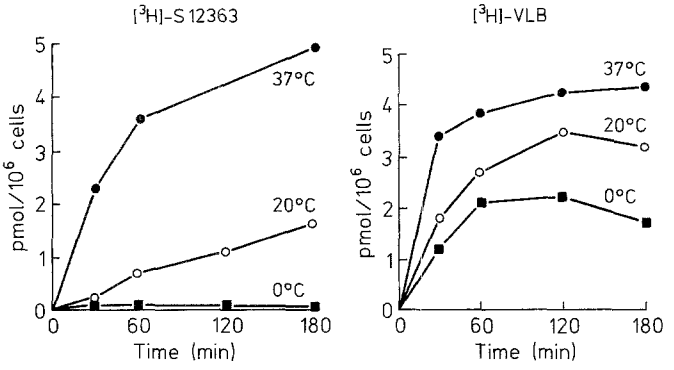


Fig. 5. Effect of temperature on the cellular accumulation of [^{3}H]-VLB (*right panel*) and [^{3}H]-S 12363 (*left panel*) by L1210 cells. The cells were incubated with 100 nM [^{3}H]-VLB or 20 nM [^{3}H]-S 12363 at the indicated temperatures and the cellular accumulation was measured

sitivity to temperature. The results presented in Fig. 4 show that the intracellular concentrations of [^{3}H]-VLB and [^{3}H]-S 12363 were globally proportional to their extracellular concentrations over the wide range of concentrations tested. The curves were not strictly linear, but rather appeared to have two slopes. However, no saturation was obtained, even at high concentrations. The effect of temperature on the accumulation of [^{3}H]-S 12363 and [^{3}H]-VLB was then studied. The results presented in Fig. 5 show that the intracellular accumulation of [^{3}H]-S 12363 was considerably more sensitive to the effect of temperature than was that of [^{3}H]-VLB.

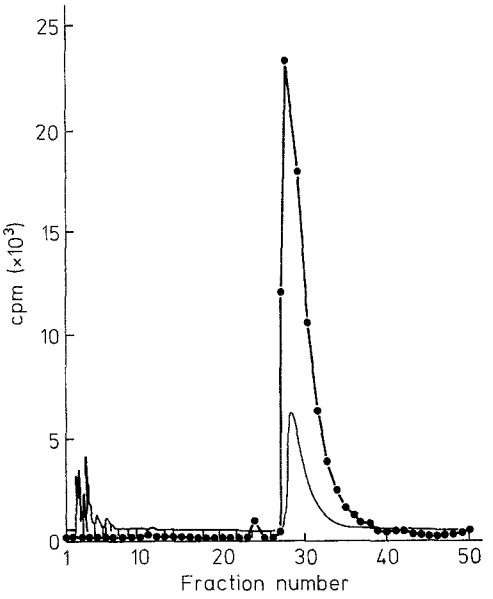


Fig. 6. HPLC analysis of L1210 cell-associated radioactivity. L1210 cells were incubated with 50 nM [^{3}H]-S 12363 for 3 h and then washed. The radioactivity was extracted and analyzed (●——●) by HPLC in the presence of S 12363, which was detected at a wavelength of 204 nm (*curve without symbols*)

HPLC analysis of cellular radioactivity

L1210 cells were incubated with [^{3}H]-S 12363 as described in Materials and methods, and the radioactivity in the cell pellet was extracted with ethanol. The radioactivity in the organic phase (nearly 100% of the total radioactivity) was then analyzed by HPLC. As shown in Fig. 6, 91% of the radioactivity was recovered at a retention time corresponding to unchanged S 12363. The intracellular radioactivity measured in the uptake and efflux experiments thus represented unmodified [^{3}H]-S 12363.

Cellular accumulation and retention by the human cell lines

To confirm the above results, we measured the same parameters in five human cell lines. The drug concentrations were selected to obtain, as far as possible, accumulation levels of the same order of magnitude. From the intracellular amount of drugs and the value obtained for the cell volume, we calculated the intracellular concentration and the corresponding accumulating factor (AF). Even after a short period of exposure (1 h), [^{3}H]-S 12363 was highly concentrated in the cells (average AF, 122), significantly more so than either [^{3}H]-VLB (average AF, 29) or [^{3}H]-VCR (average AF, 9; Table 3). The AF for [^{3}H]-S 12362 amounted to only 7.0 after 1 or 3 h exposure.

The percentage of radioactivity remaining in these cells after 3 h incubation in drug-free medium was measured. Results similar to the previous ones were obtained in all of these cell lines: the retention of [^{3}H]-S 12363 was high, being similar to that of [^{3}H]-VCR, whereas [^{3}H]-VLB and [^{3}H]-S 12362 were rapidly released from the cells (Table 3).

Table 3. Cellular accumulation and retention of tritiated compounds by L1210 and human cell lines

	Accumulation factor				Cellular retention (% of initial uptake)			
	[^{3}H]-VCR	[^{3}H]-VLB	[^{3}H]-S 12363	[^{3}H]-S 12362	[^{3}H]-VCR	[^{3}H]-VLB	[^{3}H]-S 12363	[^{3}H]-S 12362
L1210	12.0	41.0	167.0	7.0	36.7	6.1	40.2	2.1 ± 1.1
HL60	9.0	39.0	142.0		85.3	11.5	62.5	
HT-29	7.0	28.0	134.0		81.7	10.4	72.5	
COLO 320DM	14.0	19.0	105.0		33.4	1.8	43.5	
NCI-H460	9.0	21.0	130.0		36.1	7.1	66.7	
PANC-1	5.0	23.0	54.0		70.6	9.1	71.0	

The uptake of drugs was measured after 1 h incubation of cells with 400 nM [^{3}H]-VCR, 100 nM [^{3}H]-VLB, 1,000 nM [^{3}H]-S 12362, or 20 nM [^{3}H]-S 12363. The accumulation factors indicate the ratio of internal vs external concentrations (means of 2 independent values). Cells were then washed and the cellular concentration was measured after 3 h incubation in drug-free medium. The results are expressed as a percentage of the initial uptake and represent the mean of 2 values obtained in 2 independent experiments

Discussion

The aim of this study was to explain why S 12363 is considerably more potent than the other vinca alkaloids. For this purpose, we examined the interaction between S 12363 and its presumed pharmacological target, the tubulin dimer of tumor cells (L1210), and measured the cellular accumulation of this compound by tumor cells. Two of the currently used vinca alkaloids (VCR and VLB) were chosen as reference compounds together with S 12362, the inactive epimer of S 12363, to serve as a negative control.

The affinity of these compounds to tubulin from brain cytosol was first measured. The apparent K_d value for VLB was 1.2 μM, which is in agreement with previously published values [27] determined under similar experimental conditions. Affinity of the same order of magnitude was obtained for VCR (0.6 μM) and for S 12363 (1.1 μM), whereas S 12362 bound to tubulin at a lower affinity (4.9 μM). Taken together, these results are in agreement with the equipotency of VLB, VCR [10, 15], and S 12363 [23] in inhibiting brain-tubulin polymerization in vitro. Similar apparent K_d values were obtained using tubulin in crude extract from L1210 cells, suggesting that tubulin in these cells does not differ from that in brain cytosol with respect to binding of vinca alkaloids.

Binding of vinca alkaloid derivatives is a complex phenomenon that can be influenced by the degree of aggregation induced by the derivatives [21]. To date, we have gathered no information on the aggregating properties of S 12362 and S 12363 under our experimental conditions. The apparent dissociation constants are thus valid for comparative purposes only, and elucidation of the precise mode of interaction between S 12363 and tubulin requires further experimental studies.

Investigations of the uptake and retention by cells in culture of the labeled compounds indicated that the values for [^{3}H]-VCR and [^{3}H]-VLB are in agreement with previous findings [6–8, 18, 25] in that [^{3}H]-VLB entered the cells more rapidly than [^{3}H]-VCR but was less tightly retained after the cells had been washed and transferred to drug-free medium. However, the rate of accumulation of [^{3}H]-S 12363 was faster than that of [^{3}H]-VLB, and no plateau was evident after 3 h. At the same extracellular concentration (20 nM) and after 1 h incubation, the cellular accumulation of [^{3}H]-S 12363 was 4-, 18-, and 40-fold that of [^{3}H]-VLB, [^{3}H]-VCR, and [^{3}H]-S 12362, respectively.

The intracellular amount of [^{3}H]-S 12363 was strongly retained by L1210 cells, as was the case for [^{3}H]-VCR. After 3 h incubation in drug-free medium, 37%–55% ([^{3}H]-VCR) and 36%–47% ([^{3}H]-S 12363) of the initial radioactivity remained associated with the cells vs 2%–4% for [^{3}H]-S 12362 and <6% for [^{3}H]-VLB. Similar results were obtained using five human cell lines: HL60 leukemia, HT-29 and COLO 320DM colon cancer, PANC-1 pancreas cancer, and NCI-H460 lung cancer. In each cell line, [^{3}H]-S 12363 was taken up by cells more rapidly and more extensively than was [^{3}H]-VLB or [^{3}H]-VCR and was retained to the same extent as [^{3}H]-VCR.

The overall mechanism of action of S 12363 at the molecular level is probably similar to that of other vinca alkaloids as suggested by the binding to tubulin, the inhibition of tubulin polymerization [23], and the accumulation of treated cells in the M phase of the cell cycle, which was observed at concentrations corresponding to the IC_{50} [19]. In the present study, its cytotoxicity was directly related both to the intracellular concentration of the drug during the exposure of cells and to its retention after cells had been washed. The potency of S 12363 is thus probably attributable to its high cellular accumulation and retention, even after brief exposures of the cells to the drug. This was confirmed by the properties of S 12362, which displayed 30-fold lower cellular accumulation and 300-fold lower cytotoxicity than S 12363. However, it is possible that a high proportion of the S 12363 may be associated with the cell membrane or some cellular compartments. In this case, the amount of drug available for tubulin binding is lower, and the high potency of S 12363 might be due to important differences between the binding to tubulin in intact cells (for example, a higher affinity) and that observed in vitro.

Vinca alkaloids are known to be strongly concentrated by cells: the ratio of internal to external concentration (AF) can be as high as 100 during prolonged exposures [6]. Even after a short period of exposure (1 h), [^{3}H]-S 12363 was highly concentrated in the cells (average AF, 122), significantly more so than [^{3}H]-VLB (average AF, 29) or

[^{3}H]-VCR (average AF, 9). The AF value for [^{3}H]-S 12362 was only 7.0 after 3 h exposure.

The precise mechanism by which S 12363 is highly accumulated and strongly retained by tumor cells remains unknown. When S 12363 was designed, we hypothesized [17, 24] that this drug would be highly retained by tumor cells, since the metabolic conversion of the neutral phosphonic ester to a charged acid would reduce the ability of the compound to recross the membrane. Therefore, we used HPLC to analyze the intracellular radioactivity of L1210 cells that had been preloaded with [^{3}H]-S 12363 and found that 91% of the radioactivity was recovered as unchanged drug. The cellular accumulation of S 12363 is thus an intrinsic property of the unmodified molecule. A similar lack of cellular metabolism of VCR and VLB in different tumor cell lines has previously been reported [2, 3, 6].

The exact mechanism of vinca alkaloid transport in cells remains unresolved. Essentially, two models may account for the accumulation of these molecules by cells: (1) active transport, which is saturable, energy-dependent, and sensitive to temperature [3, 28, 29]; or (2) passive diffusion, which is nonsaturable but is governed by intracellular, energy-dependent binding and/or sequestration in some cellular compartment [2, 9]. The accumulation of [^{3}H]-VLB and [^{3}H]-S 12363 was nonsaturable under our experimental conditions, but the accumulation of [^{3}H]-S 12363 was more sensitive to temperature than was that of [^{3}H]-VLB. Our results are compatible with a passive-diffusion model governed by intracellular binding that would be very sensitive to temperature. However, an active transport of S 12363 conferred by the phosphonoamino acid and possessing high stereoselectivity as shown by the extremely low accumulation of [^{3}H]-S 12362 cannot be definitively ruled out at this time.

In conclusion, our results demonstrate that the potency of S 12363 is due at least in part to both the cellular accumulation and the cellular retention of the unmodified drug. The precise mechanism of this phenomenon at the molecular level remains unknown. This accumulation of S 12363 by tumor cells could confer to this drug a therapeutic advantage over its congeners. The high uptake and retention of S 12363 in solid tumor cells in culture demonstrated in the present study and the good antitumor activity observed in vivo against xenografts of these cells in nude mice [3, 4] are particularly encouraging.

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