Anti-Cancer Drugs, **1**, pp. 179–183

Flow cytometric evaluation of the cell cycle perturbations induced by S12363, a new vinca alkaloid derivative

Stéphane Leonce,[CA] Monique Anstett, Valérie Combe-Perez and Alain Pierre

The authors are at the Institut de Recherches Servier, 11 rue des Moulineaux, 92150 Suresnes, France. Tel: 33-1-45-06-51-73; Fax: 33-1-45-06-76-06; Telex: INDSERV 614097.

L1210 cells treated for 21 hours with S12363, a new vinca alkaloid derivative and the parent compounds (vinblastine, vincristine, vindesine) at equitoxic concentrations were found, by flow cytometry, to be equally accumulated in the G2 + M phase of the cell cycle. The chromatin structure of these cells was then analyzed in order to quantify with high precision the percentage of cells in mitosis. S12363 was found to accumulate, from the first hours of treatment (4–8 hours), and at lower concentrations, a higher percentage of cells in the M phase than the reference drugs. Taking into account previously published studies concerning the characteristics of vinblastine and vincristine uptake, our results are compatible with a facilitated uptake of S12363.

Key words: Cell cycle, flow cytometry, vinca alkaloid.

Introduction

S12363 is a new vinca alkaloid (VA) derivative, synthesized by grafting an optically active α-aminophosphonate at the C23 position of O4-deacetyl vinblastine. This compound was shown to be more cytotoxic *in vitro* than vinblastine (VLB), vincristine (VCR) and vindesine (VDS) against a panel of human solid tumors[1] and more active *in vivo* than VLB and VCR at doses 20-fold lower on two murine transplantable tumors.[2]

The precise mechanism of action of S12363 is not yet known. The high potency of S12363 does not seem to be due to a better interaction with the intracellular target of VA, the tubulin dimer, since the inhibition of tubulin polymerization by S12363, VLB and VCR was of similar potency.[2]

[CA] Corresponding Author

Furthermore, the inhibition of tubulin polymerization, by VA, into microtubules forming the mitotic spindle[1,3–5] induces an arrest of treated cells in mitosis.[6–8]

We have thus studied, by flow cytometry, the modifications induced by S12363 on the cell cycle of L1210 cells in culture and, more particularly, the accumulation of cells in the M phase as a function of both duration of treatment and drug concentrations compared with VLB, VCR and VDS. For such a purpose, we have used the chromatin structure analysis, a method allowing a precise quantification of cells in the M phase.[9]

Materials and methods

Cells and treatments

The L1210 murine leukemia cell line was cultured at 37°C under an atmosphere of 95% air–5% CO_2 in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS); penicillin (50 U/ml), streptomycin (50 μg/ml). 10 mM Hepes, and 2 mM glutamin (all from Gibco, Scotland).

[^{3}H]thymidine ([^{3}H]TdR) incorporation was performed as previously described.[2] Briefly, 10^5 cells/ml were incubated with the tested drugs for 24 h and then labeled with 10 μCi/ml [^{3}H]TdR for 3 hours. Cells were collected on filters and washed, and the radioactivity was then counted. Results are expressed as IC_{50}, the drug concentration which inhibited by 50% [^{3}H]TdR incorporation with respect to untreated cells.

Four compounds were tested: VLB, VDS (Eli-Lilly, France), VCR (Roger Bellon, France),

and S12363. For all experiments, L_{1210} cells were treated at the indicated factor doses of the IC50 value determined by the [^{3}H]TdR incorporation assay.

For cell cycle analysis, cells (2×10^5/ml) were incubated with the compounds for 21 h at 37°C and for 4–12 h in the case of chromatin structure analysis.

Staining of cells

Before staining, cells were centrifuged (200***g***) for 5 min at 4°C. Hoechst 33342 (HO) (Hoechst, France) and Acridine Orange (AO) (St Louis Sigma, USA) were dissolved (1 mg/ml) in distilled water and kept in the dark at 4°C.

HO staining. After centrifugation, cell pellets were suspended in complete culture medium with 7 μg/ml of HO and incubated for 1 hour at 37°C. Samples were then directly analyzed by flow cytometry.

AO staining. After centrifugation, cell pellets were fixed for 30 min at 4°C by 1 ml of ethanol at 70% (v/v). Cells were then washed twice with phosphate-buffered saline (PBS, Eurobio, France) and incubated for 1 h at 37°C with 100 μg/ml RNAse (Sigma). After centrifugation, pellets were suspended in 1 ml of solution A (0.1 M KCl, 0.1 M HCl, pH 1.4) for 30 s at room temperature, then 2 ml of solution B (0.02 M Na_2HPO_4, 0.09 M citric acid, 6 μg/ml AO) were added. Samples were then analyzed by flow cytometry.

Flow cytometry. Samples were analyzed using an ATC3000 flow cytometer (Bruker, Wissembourg, France) equipped with an argon 2025 laser (Spectra-physics, Les Ulis, France). For HO fluorescence analysis, the laser was optimized in all UV lines (351–364 nm) at 120 mW. HO fluorescence was collected through a 450 nm band-pass filter (Oriel, Paris, France) and 20 000 cells were measured for each DNA histogram.

For AO fluorescence analysis, the laser was optimized at a wavelength of 488 nm at 400 mW. The two AO fluorescences (green and red) were separated by a 570 nm dichroic long-pass filter and collected through two band-pass filters (Oriel) (520 nm for green fluorescence and 630 nm for red fluorescence). For each sample, 5000 cells were analyzed at a speed of 1000 events per second. Information was collected in 'list-mod' and displayed as cytograms (single-stranded DNA, red fluorescence; double-stranded DNA, green fluorescence). Percentages of cells in the M phase were calculated by using a window defined, through the computer, around the identified population.

Results are expressed either as the percentage of cells in the considered phase of the cell cycle, or as the increase of this percentage induced by the treatment with respect to untreated control cells.

Results

[^{3}H]TdR incorporation

The IC_{50} values of VLB, VCR, VDS, and S12363, estimated by the inhibition of [^{3}H]TdR incorporation, were respectively 3.10 nM, 4.85 nM, 6.50 nM, and 0.43 nM (means of at least two values).

Cell cycle analysis by HO staining

Untreated, exponentially growing L1210 cells have a characteristic cell cycle with approximately 37% of cells in the G0–G1 phase, 50% in the S phase, and 13% in the G2 + M phase.

After being treated for 21 h by the VA derivatives, L_{1210} cells were massively accumulated in the G2 + M phase. Figure 1 shows the

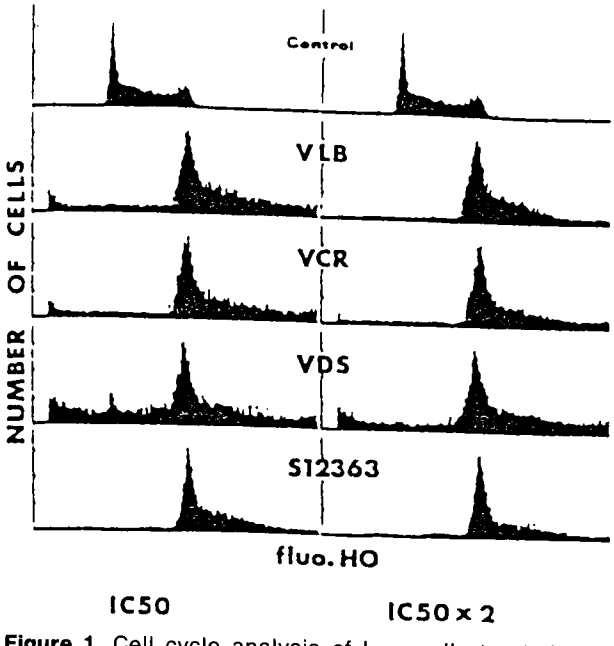

Figure 1. Cell cycle analysis of L_{1210} cells treated with vinca alkaloid derivatives. L_{1210} cells were exposed to the compounds at concentrations corresponding to the IC_{50} (left panels) and $IC_{50} \times 2$ (right panels), during 21 h. Cells were then washed, stained by HO and analyzed as described under Materials and methods.

modification of the cell cycle induced by the drugs tested at the IC_{50} and $IC_{50} \times 2$ concentrations. We quantified, at a concentration corresponding to the IC_{50} value, an increase of G2 + M cell percentage ranging from about 60% to 70% (including polyploid cells) with respect to untreated cells, except for VDS (50% to 60%). However, small differences in percentage are not easy to quantify precisely by this staining procedure. It is only possible to determine the lowest amount of debris in S12363-treated cells and the lowest accumulation in G2 + M in VDS-treated cells. The percentage of cells with an amount of DNA superior to G2 + M phase is similar for all tested compounds (40%).

Chromatin structure analysis by AO staining

The L_{1210} cell line has a doubling time of 10–12 h, and the percentage of cells in the M phase is low, generally ranging from about 2% to 5%. Figure 2 shows the distribution of exponentially growing L_{1210} cells in the cell cycle after an AO double- and single-stranded DNA staining. Cells in the M phase are easily identified and quantified.

Cells were then treated with the drugs at three different concentrations corresponding to $IC_{50}/2$, IC_{50} and $IC_{50} \times 2$. Figure 3 shows the variations in the percentages of cells in the M phase as a

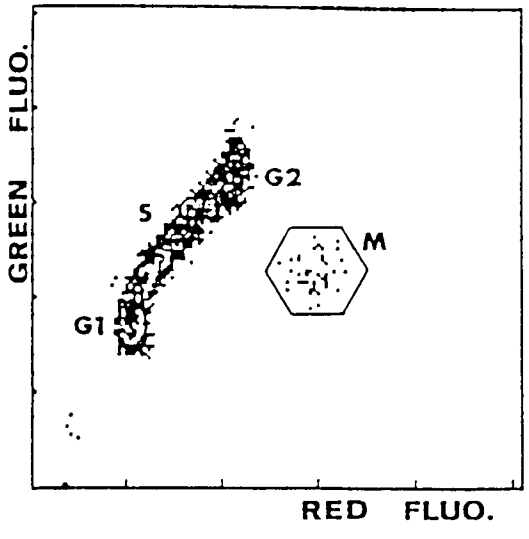

Figure 2. Chromatin structure analysis of untreated L_{1210} cells. L_{1210} cells were stained with AO after a partial acid denaturation of the DNA. Green and red fluorescences were analyzed by flow cytometry as described under Materials and methods.

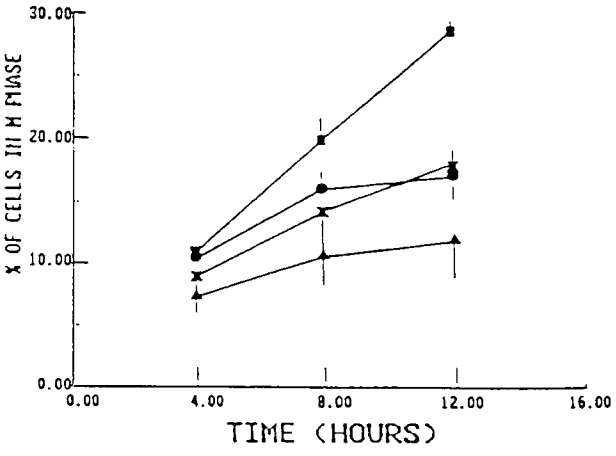

Figure 3. Variation of M cell percentages as a function of time of treatment. L_{1210} cells were exposed to S12363 (■–■), VLB (●–●), VCR (▲–▲), VDS (X–X) at the IC_{50} values. Aliquots of cells were sampled at the times indicated (4, 8, 12 h) and percentages of M phase cells were determined by flow cytometry as described under Materials and methods. Each point represents the mean of three independent experiments. Bars, SE.

function of the incubation time at the IC50 concentration. It clearly appears that, from the first hours of treatment (4–8 h), S12363 accumulated a larger percentage of cells in the M phase than the other drugs. After 12 h of treatment, 28.75% of S12363-treated cells were in the M phase, compared with 17.15% (VLB), 17.68% (VDS), and 12.05% (VCR). Similar results were obtained with cells treated by drug concentrations corresponding to $IC_{50} \times 2$ (data not shown). On the contrary, no differences in M phase accumulation were observed between the drugs for a continuous exposure (21 h, data not shown).

Figure 4 shows the variation of the percentage of cells in the M phase as a function of drug concentration after an incubation time of 12 h. Important differences in accumulation between drugs were observed: S12363 induced a significant increase (13.75%) of the percentage of cells in the M phase at low doses ($IC_{50}/2$), while the reference compounds did not change cell cycle pattern. The differences are sharper at higher doses (IC_{50}): 29.20% of cells treated by S12363 were in the M phase, while the percentages obtained with the reference compounds were only 13.40% (VLB), 13.00% (VDS) and 6.65% (VCR).

Discussion

In the present work, we describe the L_{1210} cell cycle perturbations induced by S12363 in compar-

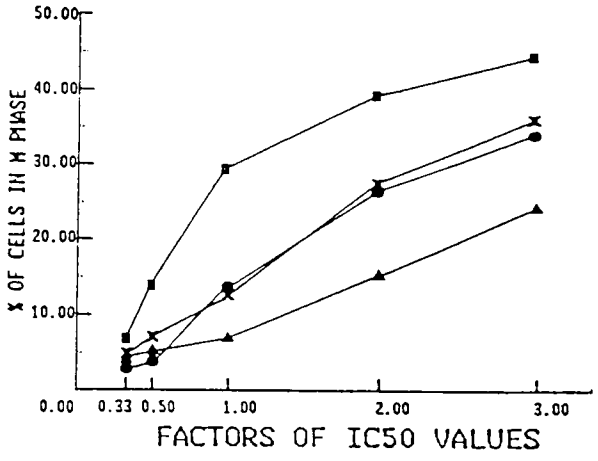

Figure 4. Variation of M phase cell percentage as a function of drug concentrations. L_{1210} cells were exposed to S12363 (■–■), VLB (●–●), VCR (▲–▲), VDS (X–X) during 12 h at different concentrations (from $IC_{50}/3$ to $IC_{50} \times 3$ value) and percentages of M phase cells were determined by flow cytometry.

ison with the reference compounds VLB, VCR and VDS.

S12363 was 7–15-fold more cytotoxic than the reference compounds. In the case of VCR and VLB, the concentration × time values (0.064 μg h/ml and 0.174 μg h/ml, respectively) obtained in patients[10] are of the same order of magnitude as our IC_{50}. Although no pharmacokinetic data are yet available for S12363, we can hypothesize that these effects observed *in vitro* can be extrapolated to clinical situations.

Using the HO technique to stain DNA, we have shown that all the drugs, when tested at equitoxic concentrations and for a long incubation time (21 h), have massively accumulated the cells in the G2 + M phase (from 63.8% for VLB to 69.6% for S12363 at the IC_{50} values). Only VDS was slightly less active (49.8%). On the contrary, we observed that S12363 provoked lower cellular debris than the other compounds. The latter observation suggests that S12363, in these experimental conditions, has a lower lytic activity than VLB, VCR, and VDS.

VA derivatives are known to arrest cell division in mitosis presumably by inhibition of tubulin polymerization.

The analysis of the chromatin structure, by the AO staining method, has allowed us to quantify this accumulation of cells in mitosis. Although all the drugs were used at equitoxic concentrations, we found that S12363 accumulated, from the first hours of treatment, a larger percentage of cells in the M phase than VLB, VDS, or VCR. After 12 h, at a concentration corresponding to the IC_{50} value, 29.20% of S12363-treated cells were in the M phase, vs 13.40% for VLB, 13.00% for VDS, and 6.65% for VCR. The latter compound is less active in this kinetic study. Furthermore, the high accumulation induced by S12363 occurred at concentrations lower than the equitoxic concentration, when the analysis was performed after 12 h (one doubling time) of treatment.

S12363, in addition to its higher potency, has the property of accumulating cells in mitosis more rapidly than VLB, VCR, and VDS. Hence this compound should be active even after a short time of contact with the tumor cells, but this property does not seem to be due to a better interaction of S12363 with the tubulin.[2]

Some recent studies suggest that the different pharmacological properties of VLB and VCR might be due, at least in part, to their different uptake and/or cellular retention.[11–12] VLB was shown to enter and to be released from cells in culture more rapidly than VCR. When cells are exposed continuously to these two drugs, no differences in cytotoxicity and in G2 + M accumulation were observed because the intracellular concentrations (at the plateau) of these two drugs appeared to be similar.

Our results obtained from the chromatin structure analysis are in agreement with these observations: VLB and VCR, which have comparable IC_{50} values (24 h exposure), accumulated the same percentage of cells in the M phase for a continuous exposure. Moreover, our kinetic experiments have shown that VLB induced a higher percentage of cells in the M phase than VCR for shorter lengths of treatment. The most logical explanation is that, at these treatment times, VLB enters the cells more rapidly and leads to a higher intracellular concentration than VCR.

Cells treated with S12363 at an equitoxic concentration were accumulated in mitosis twice as much as VLB-treated cells. Thus, as mentioned for the difference between VLB and VCR,[11,12] an obvious explanation is that the rate of S12363 intracellular accumulation is higher than that of VLB.

However, equitoxic concentrations of S12363 are 10-fold lower than those of VLB and VCR. S12363 could present, in addition to a faster rate of penetration, a higher intracellular concentration at the plateau. Thus toxic intracellular amounts of this drug could be obtained at lower extracellular concentrations, which may confer a therapeutic advantage over already used VAs.

Conclusion

Using flow cytometry, we have shown that S12363, which is highly potent *in vivo* and *in vitro*, induced, for a continuous exposure, the same accumulation of cells in the G2 + M phase as reference drugs. But it appeared to block more rapidly and for lower concentrations a higher percentage of cells in mitosis than standard VAs, suggesting a better uptake of this compound. This property might be of clinical interest for the synchronization of tumor cells which could be obtained after a brief administration of the drug. This compound is currently in phase I.

Acknowledgements

The authors would like to thank Dr G. Atassi, Dr J.A. Boutin, and Dr T. Verbeuren for their help and their critical comments on this report.

References

1. Pierre A, Leonce S, Anstett M, Hautefaye P, Lavielle G, Cudennec CA. Cytotoxic properties of new potent Vinca-alkaloid derivatives on human solid tumors, *in vitro*. *Proc Am Assoc Cancer Res* 1989; **30**: 581.
2. Pierre A, Lavielle G, Hautefaye P, Seurre G, Leonce S, Saint-Dizier D, Boutin JA, Cudennec CA. Pharmacological properties of a new α-aminophosphonic acid derivative of vinblastine. *Anticancer Res* 1990; **10**: 139–44.
3. Himes RH, Kersey RN, Beller-Betinger I, Samson FE. Action of vinca-alkaloids vincristine, vinblastine, and desacetyl vinblastine amide on microtubules *in vitro*. *Cancer Res* 1976; **36**: 3798–802.
4. Bowman LC, Houghton JA, Houghton PJ. Formation and stability of vincristine–tubulin complex in kidney cytosols (roles of GTP and GTP hydrolysis). *Biochem Pharmacol* 1988; **37**: 1251–7.
5. Jordan MA, Margolis RL, Himes RH, Wilson L. Identification of a distinct class of vinblastine binding sites on microtubules. *J. Mol Biol* 1986; **187**: 61–73.
6. Maria-Chirife A, Studzinski GP. Definition of the cell cycle segment with special sensitivity to vinblastine. *Proc Exp Biol Med* 1978; **157**: 206–10.
7. Madoc-Jones H, Mauro F. Interphase action of vinblastine and vincristine: differences in their lethal action through the mitotic cycle of cultured mammalian cells. *J Cell Physiol* 1968; **72**: 185–96.
8. Howard SMH, Theologides A, Sheppard JR. Comparative effects of vindesine, vinblastine and vincristine on mitotic arrest and hormonal response of L_{1210} leukemia cells. *Cancer Res* 1980; **40**: 2695-700.
9. Darzynkiewicz Z, Traganos F, Kimmel M. Assay of cell cycle kinetics by multivariate flow cytometry using the principle of stathmokinesis. In: Gray JW, Darzynkiewicz Z, eds. *Techniques in Cell Cycle Analysis*, Clifton: Humana Press, 1986: 291–336.
10. Davis LE, Alberts DS, Plezia PM, Roe DJ, Griswold DP. Predictive model for plasma concentration-versus-time profiles of investigational anticancer drugs in patients. *J Natl Cancer Inst* 1988; **80**: 815–19.
11. Ferguson PJ, Cass CE. Differential cellular retention of vincristine and vinblastine by cultured human promyelocytic leukemia HL60/CI cells. The basis of differential toxicity. *Cancer Res* 1985; **45**: 5480–8.
12. Gout PW, Noble RL, Bruchowski N, Beer CT. Vinblastine and vincristine: growth inhibitory effects correlate with their retention by cultured Nb2 node lymphoma cells. *Int J Cancer* 1984; **34**: 245–8.

(Received 5 July 1990; accepted in revised form 23 October 1990)