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Cytotoxicity, Cell Cycle Kinetics and Morphonuclear-induced Effects of *Vinca* Alkaloid Anticancer Agents

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Abstract

The effects of four *Vinca* alkaloids (vinblastine, vincristine, vindesine and vinorelbine) on three neoplastic cell lines (the MXT mouse mammary cell line and the T24 and J82 bladder cell lines) were studied at three biological levels, i.e. cell proliferation, cell cycle kinetics and morphonuclear characteristics. These effects were studied by means of digital cell image analysis on Feulgen-stained nuclei. The aim of the present work was to characterize the effects specifically induced by *Vinca* alkaloids as compared with those obtained previously with other pharmacological classes of anticancer drugs.

The results show that *Vinca* alkaloids inhibit the cell proliferation of neoplastic cell lines at a concentration of 10^{-8} M except in the case of the J82 cell line, for which only a slowing down of cell proliferation was observed. Concerning the cell cycle kinetics, the results show that the *Vinca* alkaloids induce an accumulation of cells in the mitosis phase. This accumulation of mitotic cells was maximal after 15 h incubation in the presence of the drugs. A study of the morphonuclear-induced effects of *Vinca* alkaloids showed that the variance of the optical density (VOD) is strongly influenced by these *Vinca* alkaloids. The development of the VOD was parallel with the development of the percentage of mitosis; thus, the VOD enabled the *Vinca* alkaloid-induced effects to be specifically characterized from a morphonuclear point of view. On the other hand, the results show that the mean value of the variance of the optical density was very highly correlated ($P < 0.001$) with the efficiency of the *Vinca* alkaloids in terms of cytotoxicity.

In clinical studies, the analysis of the development of this parameter would make it possible to assess the response to chemotherapy in the case of patients treated with *Vinca* alkaloids.

For many years, the cytoplasmic microtubule has been acknowledged as an important target in cancer chemotherapy (Kaye 1991). Historically, the first antineoplastic drugs shown to act against the microtubule were *Vinca* alkaloids. These compounds were subsequently subjected to clinical trials. These *Vinca* alkaloid derivatives (vinblastine, vincristine, vindesine and vinorelbine exert their antineoplastic activity by preventing the tubulin polymerization from forming microtubules. Another class of compound has recently been used in this way in clinical practice (Carmichael 1994). This class includes natural taxol and hemi-synthetic taxotere compounds. These agents act by inhibiting microtubule depolymerization. At present, pharmacologists use a test relating to the inhibition of the microtubule assembly to characterize the antitumoural effect of investigational agents belonging to the above-mentioned classes (Bai et al 1992, 1993; de Ines et al 1994). The mechanism of action employed by these anticancer agents is usually studied by means of immunofluorescence using tubulin-specific antibodies (Binet et al 1989; Jordan et al 1991; Bai et al 1992, 1993; de Ines et al 1994) or by means of turbidimetrical methods enabling the tubulin polymerization to be monitored (Bai et al 1992, 1993; de Ines et al 1994). The identification of agents acting at the tubulin level by means of a computer-assisted evaluation of differential cytotoxicity data has also been proposed (Paull et al 1992).

An original method enabling the rapid identification of a specific class of spindle poisons (the *Vinca* alkaloid anticancer drugs) to be carried out is described here, and the morphonuclear-induced effects of *Vinca* alkaloids are characterized in the present work.

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Materials and Methods

Drugs

Vinblastine and vincristine were from Sigma Chemical Co. (St Louis, USA), vindesine was from Eli Lilly (Brussels, Belgium) and vinorelbine was from Pierre Fabre Médicaments (Castres, France). All drugs were dissolved in phosphate-buffered saline (PBS, Gibco, Paisley, UK) at the time of the experiment.

Cell culture

The MXT cell line was established in-vitro (Kiss et al 1986) from the MXT mouse mammary adenocarcinoma (Watson et al 1977). The T24 (HTB 4) and J82 (HTB 1) (O'Toole et al 1978) cell lines, which originated in two human bladder cancers, were from the American Type Culture Collection (Rockville, USA). The three cell lines were cultured as previously described (Pauwels & Kiss 1991). Briefly, the cells were cultured at a temperature of 37°C in an

atmosphere containing 5% CO_2. The culture medium consisted of Eagle's Minimum Essential Medium supplemented with 5% foetal calf serum, L-glutamine and antibiotics (all constituents from Gibco).

Preparation of biological samples

The preparation of the biological samples was described previously (Pauwels & Kiss 1991). For each cell line, 2·5 mL culture medium containing 10 000 cells mL^{-1} were plated in 35-mm Petri dishes (Nunc, Roskilde, Denmark) containing an 18 × 18 mm microscope coverslip on which the cells were able to adhere. After 24 h incubation, the medium was supplemented with 0·2 mL PBS in the case of the control conditions, and with 0·2 mL of a solution of each of the four *Vinca* alkaloids dissolved in PBS to a final concentration of 10^{-8} M. After 2, 5, 10, 15, 24, 36, 48 and 72 h each coverslip was fixed in a mixture of ethanol 96% (75 vols), formol 40% (20 vols) and acetic acid (5 vols) (EFA), and mounted on a microscope slide. These slides were stained with the Feulgen reagent (Fluka, Buchs, Switzerland) after they had been subjected to hydrolysis in 6 M HCl for 1 h (Kiss et al 1993). This staining was DNA-specific and quantitative. Each experimental condition was executed in triplicate.

Cell growth assessment

The number of cells present in an area of 16 mm^2 was counted for each slide analysed, as previously described (Pauwels et al 1994). This was carried out using a microscope equipped with a 100-square grid. Five 16-mm^2 areas were analysed per slide. The values so obtained enabled the cytotoxic effects of the *Vinca* alkaloids on the proliferation of the cell lines to be recorded.

Image analysis

Image cytometry was performed using a Samba 2005 microscope image processor (Alcatel-TITN, Grenoble, France) with a 40 × magnification lens. Nine hundred cell nuclei were analysed for each experimental condition studied. Each cell nucleus was characterized by 15 morphonuclear parameters belonging to four groups, as follows.

The first group consisted of one geometric parameter. This parameter was the nuclear area (NA) and quantitatively describes nuclear size.

The second group consisted of five densitometric parameters. These were computed on the basis of the values of the optical density of each pixel corresponding to the nucleus. The integrated optical density (IOD) corresponds to the sum of the values of the optical density corresponding to each pixel; the IOD assesses nuclear DNA content. The remaining four parameters quantitatively describe chromatin texture from a densitometric point of view. These parameters are the mean optical density (MOD), the skewness (SK) and kurtosis (K) indices, and the variance of the optical density (VOD). The third and fourth groups also included parameters which quantitatively describe the chromatin pattern.

The third group included five parameters derived from the length section matrices. These were the run length percentage (RLP) which corresponds to the number of run lengths for a given area, the frequency of short (SRL) and long (LRL) run length chromatin clumps, which are representative of small and large chromatin clumps, respectively, their distribution (RLD) and the grey-level distribution (GLD).

The remaining four parameters were computed on the basis of co-occurence matrices. These parameters were the local mean (LM), energy (E) and the coefficient of variance (CV), which measure the level of overall chromatin condensation, and contrast (C), which is representative of the number of boundaries between nuclear regions with distinct optical densities.

Statistical and mathematical analyses

The assessment of cell growth, cell kinetic effects, and morphonuclear characteristics are reported as means (± s.e.m.) and statistically compared by means of the Fisher test (one-way analysis of variance).

As previously described (Gozy et al 1993; Pauwels et al 1994), the assessment of the proportion of cells in the various phases of the cell cycle was carried out using linear discrimination analysis with reference to specific morphonuclear data banks corresponding to the G_0–G_1, S, G_2 and M phases of the cell cycle.

Principal-components analysis followed by the canonical transformation of the data was used to characterize typical cell nuclei from control and *Vinca* alkaloid-treated cells, according to the formula which we had previously adjusted to study other pharmacological classes of antineoplastic drugs (Pauwels & Kiss 1991; Pauwels et al 1994). The correlation coefficients calculated in the present work corresponded to the Kendall and Pearson correlation coefficients.

Results

Cell proliferation

Fig. 1 describes the cell proliferation in the different experimental conditions studied. The data show that the four *Vinca* alkaloids are significantly ($P < 0·001$) effective against the three cell lines tested. The cytotoxic effects of these drugs appeared early, especially in the case of the MXT cell line, for which the toxic action was statistically significant as early as 5 h after the beginning of the treatment. In the case of the T24 cell line, the cytotoxic effect became significant 15 h after the beginning of the treatment. The effects of all the *Vinca* alkaloids on the J82 cell line were only statistically significant from the 36th hour after the beginning of the treatment.

The efficiency of the drugs was similar with respect to any given cell line. When the sensitivity of the cell lines were compared, the results showed that the cell proliferation of the MXT and T24 cell lines was completely eliminated, though the results showed only a slowing down of the proliferation in the J82 cell line. Due to the marked effect of the *Vinca* alkaloids on the cell proliferation of the MXT and T24 lines, the cell nuclei surviving 36 h after the addition of the drugs were not analysed because they were too few for a reliable morphonuclear analysis. For these reasons, the figures depicting the cell cycle kinetics (Fig. 2) and the development of the optical density variance (Fig. 3) of the MXT and T24 cell lines include only the results obtained during the first 24 h of incubation.

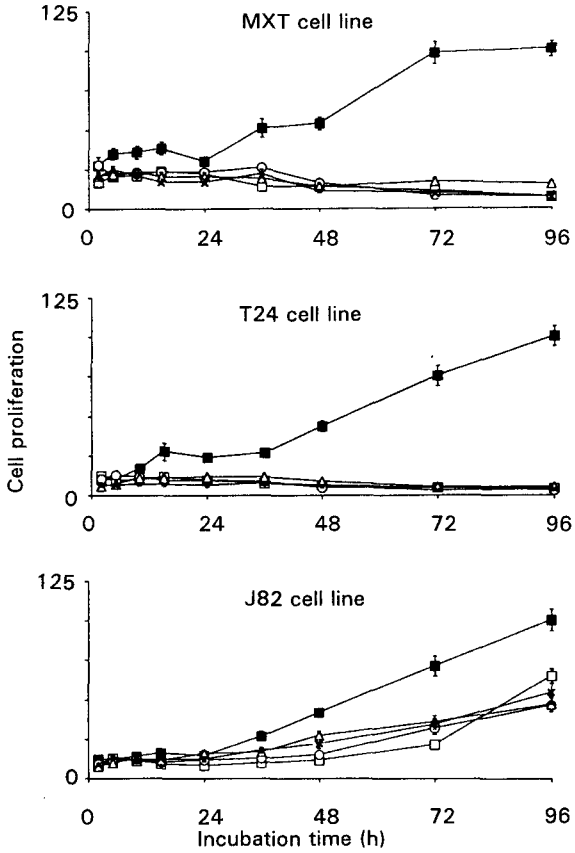

FIG. 1. Cell proliferation of the MXT, T24, and J82 cell lines after their treatment with vinblastine (□), vincristine (○), vindesine (△) or vinorelbine (×). Cell proliferation of the cell lines without the addition of any drugs ■. Results are expressed as means ± s.e.m.

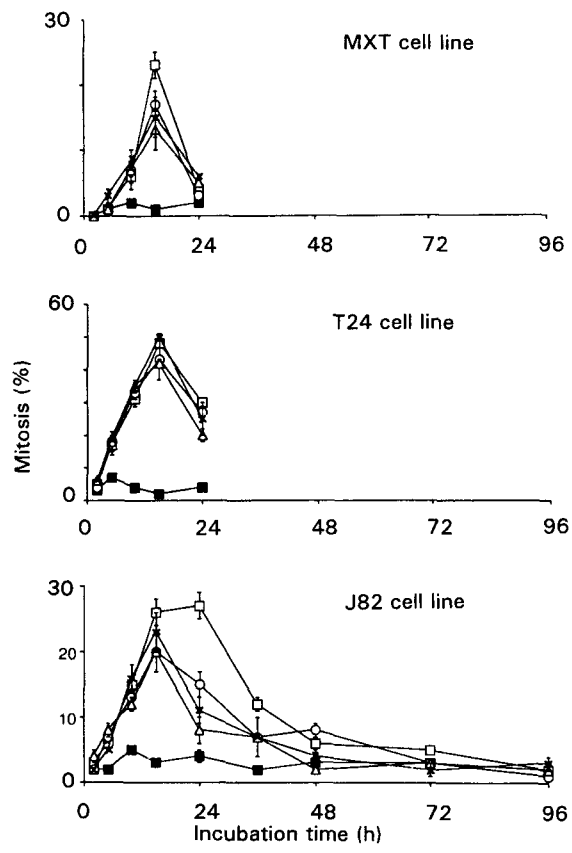

FIG. 2. Development of the percentage of mitosis during the incubation of the MXT, T24 and J82 cell lines in the presence of vinblastine (□), vincristine (○), vindesine (△) or vinorelbine (×). Mitosis of the cell line without the addition of any drugs ■. Results are expressed as means ± s.e.m.

Cell cycle kinetics

Fig. 2 gives the percentage of mitosis in the different experimental conditions studied. The data show that the *Vinca* alkaloids induced significant modifications in the cell cycle kinetics from the 5th to the 15th hour after the beginning of the treatment in the case of the MXT cell line, and from the 2nd to the 24th hour in the case of the T24 and J82 lines. The maximum percentage of mitosis was observed 15 h after the addition of the *Vinca* alkaloids. One exception was observed, i.e. the case of the J82 cell line, where vinblastine induced a higher percentage of mitotic cells at the 24th hour. Nevertheless, the mitosis percentages were not significantly different after the 15th and the 24th hours of incubation in the case of the J82 cell line.

Multivariate analysis

Multivariate analysis taking account of the 15 morpho-nuclear parameters was performed to study the influence of each of the 15 parameters in only one calculation step. For each cell line, the experimental conditions which corresponded to the most important effects in terms of cell kinetics (i.e. the experimental conditions obtained after 15 h incubation) were submitted to principal-components analysis; for a given cell line, the 900 cell nuclei corresponding to each experimental condition (i.e. the drug-treated cells) obtained after 15 h incubation in the presence or absence of 10^{-8} M of the drug were projected into the two-dimensional space defined by the first two canonical functions (canonical projection of the data).

Fig. 3 shows the results so obtained. The ellipses in charts A, C and E represent the 95% confidence interval around the mean position of the factorial cell distribution of the different nuclear populations. The data show that for each cell line studied, the nuclear populations corresponding to the *Vinca* alkaloid-treated cells were located on the left of the first canonical function (usually under the negative values of this canonical function), although the non-treated cells were located under the positive values of this first function. Charts B, D and F indicate the parameters having the most important influence on the first canonical function (i.e. the canonical function discriminating between the *Vinca* alkaloid-treated conditions compared with the control conditions). The most discriminating parameters were the integrated optical density (IOD) (MXT cell line), the mean optical density (MOD) (T24 and J82 cell lines) and the variance of the optical density (VOD) (MXT, T24 and J82 cell lines), i.e. three parameters describing the nuclear texture from the densitometric point of view. Of these three parameters, the variance of the optical density appeared to be the best parameter for discriminating between the *Vinca* alkaloid-treated cells and the control cells. For each cell line,

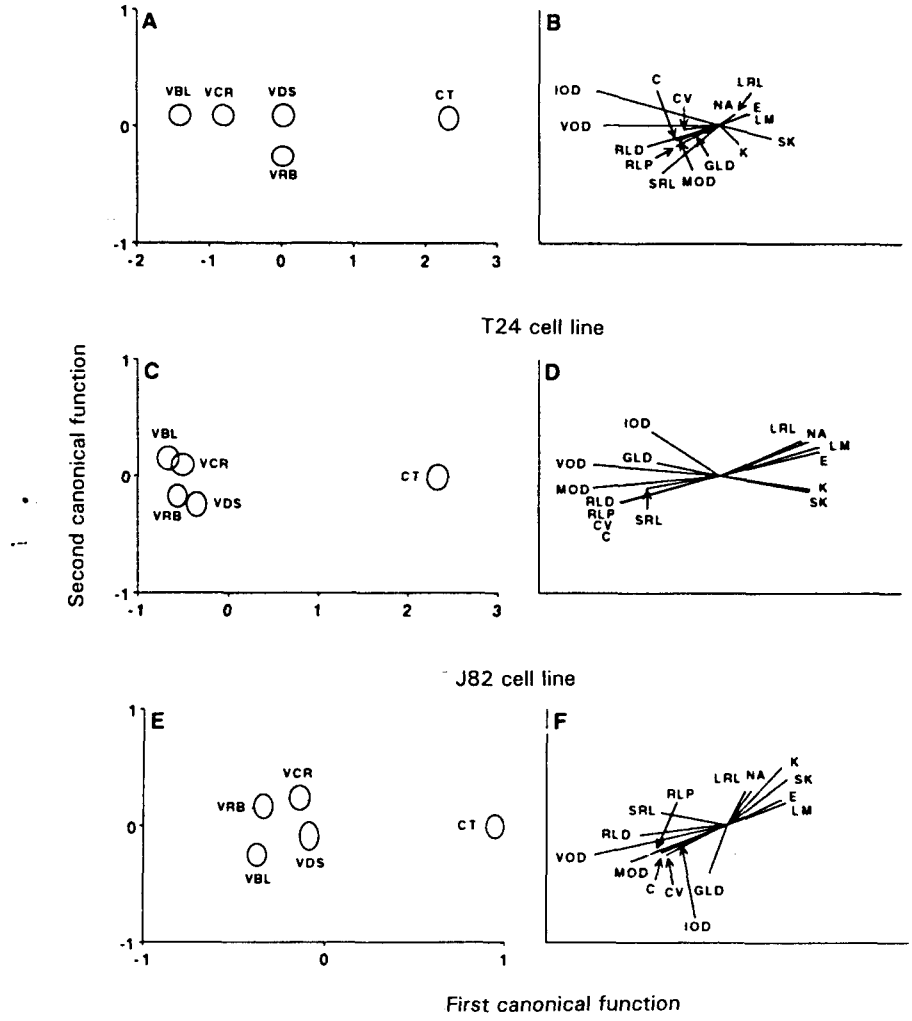

FIG. 3. Results obtained after performing multivariate analyses on the drug-induced morphonuclear modifications recorded on 900 nuclei from merged files containing the digitized nuclear images for the drug-treated MXT (A and B), T24 (C and D) and J82 (E and F) cell lines. Each nucleus is described by means of 15 morphonuclear parameters and submitted to principal-components analysis. The nuclei were thus located in a 15-dimensional space on the basis of their 15 nuclear parameters. They were then projected into a two-dimensional space by the canonical transformation of their data. This two-dimensional space is defined by the first two canonical functions. Charts A, C and E represent complex multifactorial functions featuring the nuclei. Each ellipsis represents the 95% confidence interval around the mean position of the factorial cell distribution. Charts B, D and F represent the canonical projection for the 15 parameters. This projection indicates the direction and strength of the parameter scattering for the cell populations under analysis. VBL = vinblastine, VCR = vincristine, VDS = vindesine, VRB = vinorelbine.

the optical density variance was in fact the only parameter which appeared among the two best parameters according to the first canonical function.

Monovariate analysis

Because the variance of the optical density was the parameter which best discriminated between the *Vinca* alkaloid-treated and the non-treated cells, the development of the mean values of this parameter is represented in Fig. 4 for the different experimental conditions studied. The development of the mean variance of the optical density (VOD) values (Fig. 4) is similar to that obtained with the mitosis percentage (Fig. 2). Thus, the mean VOD values increased immediately after the beginning of the treatment, i.e. as early as the 10th hour after the addition of the drug in the case of the MXT and J82 cell lines, and the 5th hour in the case of the T24 cell line. This mean VOD value increased to reach a maximum after 15 h incubation in the presence of the drugs.

Bivariate analysis

The results illustrated in Fig. 4 show that the maximum mean values of the optical density variance observed in the case of the drug-treated conditions attained arbitrary values above 15 in the case of the T24 cell line, below 12 in the case of the J82 line, and values which were intermediate in the case of the MXT line. An analysis of Fig. 1 reveals that the efficiency of the *Vinca* alkaloids was maximal with the T24 cell line (at least 95% of the T24 cells were killed after 96 h incubation). On the other hand, the *Vinca* alkaloids killed

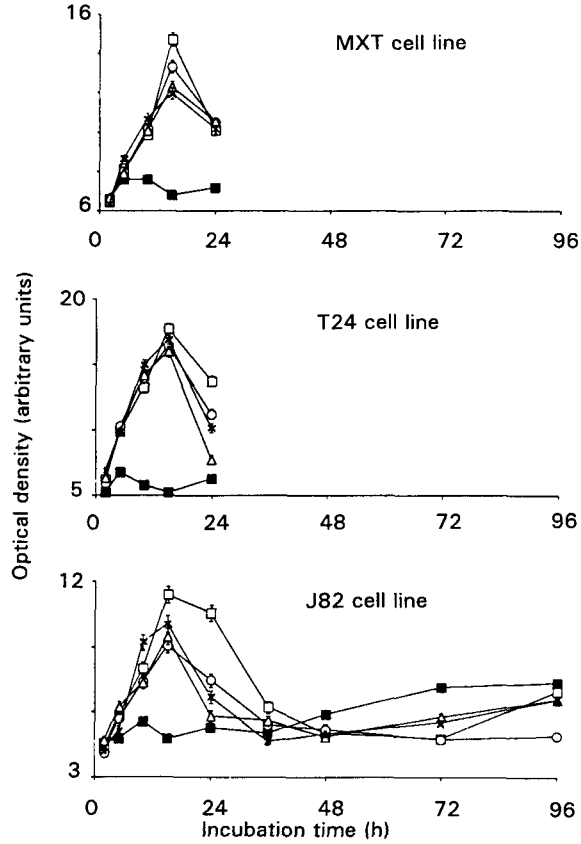

FIG. 4. Development of the mean value of the variance of the optical density parameter during the incubation of the MXT, J82 and T24 cell lines in the presence of vinblastine (□), vincristine (○), vindesine (△) or vinorelbine (×). Optical density in cell lines without drug, ■. Results are expressed as means ± s.e.m.

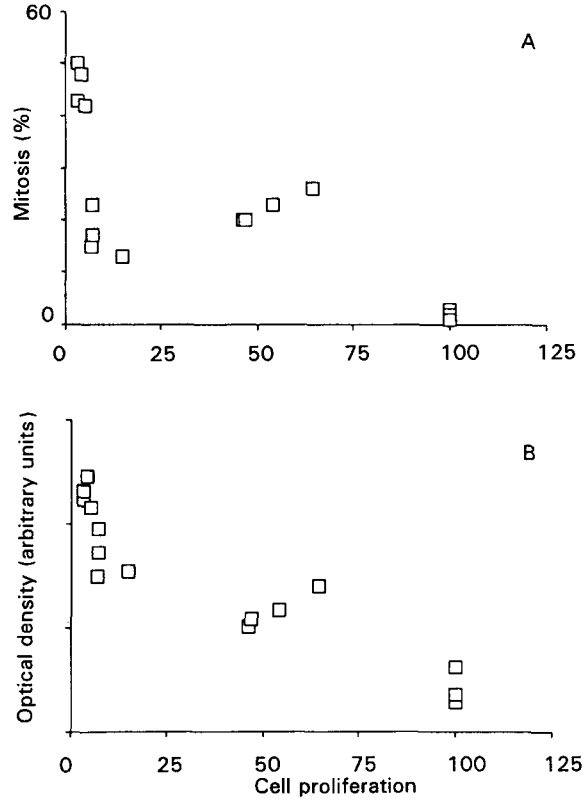

FIG. 5. Correlations obtained between the cell proliferation and the percentage of mitosis (A) on the one hand, and the mean value of the optical density variance (B) on the other. The percentage of mitosis and the mean value of the optical density variance were calculated after 15 h incubation in the presence of the drugs; the cell proliferation was calculated after 96 h of incubation in the presence of the drugs. The open squares correspond to the different experimental conditions obtained with the three cell lines and the four *Vinca* alkaloids, or in the absence of any drugs (control).

fewer than 60% of the T24 cells. In the case of the MXT cells, the percentages killed by the drugs are included between those of the T24 and J82 cell lines. Thus, it appears that there is a correlation between the effects of the *Vinca* alkaloids on cell proliferation and optical density variance. The correlation existing between cell proliferation and the variance of optical density was studied by means of the Kendall non-parametric test. This correlation was studied because if such a correlation is statistically significant, it will help clinicians to evaluate the efficiency of *Vinca* alkaloid treatments. As the percentage of mitosis and the mean VOD values described similar developments, the correlation between cell proliferation and the percentage of mitosis was also studied.

Correlations obtained between cell proliferation and the percentage of mitosis or the mean VOD values are shown in Fig. 5. Using the Kendall test, the correlation coefficients obtained were $-0{\cdot}55$ ($P < 0{\cdot}01$) and $-0{\cdot}75$ ($P < 0{\cdot}001$) (Figs 5A and 5B, respectively). Since the best correlation was obtained with cell proliferation and the mean VOD values, we chose to study the linearity of this relation. The Pearson correlation coefficient was calculated to this end. Such a coefficient has a value of -0.92 ($P < 0{\cdot}001$). This result indicates that a linear relation exists between cell proliferation and the mean VOD values.

Discussion

Vinca alkaloids have been used since the sixties in cancer chemotherapy. At present, the four *Vinca* alkaloids used in this study are usually employed in chemotherapy (Zhou & Rahmani 1992). In the present work we studied the effects of these four anticancer drugs on the morphonuclear features of three neoplastic cell lines. Other studies relating to other pharmacological classes of anticancer drugs and using the same methodology are described elsewhere (Pauwels & Kiss 1993; Pauwels et al 1994). With respect to these other pharmacological classes, the drugs led to important modifications in the morphonuclear parameters, such as an increase in the nuclear DNA content (explained by the accumulation of cells in the S or G_2 phases), with a consequent increase in nuclear size. These phenomena were accompanied by an increase in the frequency of the large dense chromatin clumps in the nuclei and consequently by an increase in the overall chromatin condensation (Pauwels et al 1994).

The results obtained in this work show that at a concentration of 10^{-8} M, the *Vinca* alkaloids had an important cytotoxic effect on the MXT and T24 cell lines. This phenomenon is a well-known effect of *Vinca* alkaloids (Tucker et al 1977; Jordan et al 1991; Zhou &

Rahmani 1992). On the other hand, cell proliferation in the J82 cell line only slowed down under the action of these anticancer drugs. This phenomenon is explained by the fact that this cell line possesses a drug expulsion mechanism limiting the effects of some drugs (Delville et al 1995).

The results of principal-components (multivariate) analysis show that the optical density variance was the morphonuclear parameter providing the most discrimination between untreated cell nuclei and *Vinca* alkaloid-treated cell nuclei. Previous work has shown that this parameter rarely discriminates between control cell nuclei and cell nuclei treated with antimetabolites, or alkylating and intercalating agents (Pauwels & Kiss 1993; Pauwels et al 1994). The very significant influence of the *Vinca* alkaloids on the optical density variance is due to the fact that this parameter discriminates best between mitotic features and the nuclear features of the other cell cycle phases (Colomb & Martin 1992). The analyses of the development of the percentage of mitotic features (Fig. 2) and the development of the mean values of the optical density variance (Fig. 4) confirm the similarity between these two parameters. It appears that optical density variance is a good marker because it is highly specific to the action of *Vinca* alkaloids. A significant consequence of this observation is that the optical density variance is a possible parameter enabling the efficacy of *Vinca* alkaloid treatment to be evaluated. This hypothesis is confirmed by the correlation shown in Fig. 5B: indeed, it appears that the mean values of the optical density variance after 15 h incubation in the presence of the drugs correlated strongly with the efficacy of the treatment. It has been previously demonstrated that the increase in the nuclear area (which is a measure of nuclear size) is a possible parameter enabling the response to chemotherapy to be predicted (at least in-vitro) (Pauwels & Kiss 1995). Unfortunately, this observation was not respected in the case of the *Vinca* alkaloids. The present work demonstrates that the study of the optical density variance makes it possible to fill this gap.

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