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Morphonuclear characterization of drug resistance by means of digital cell-image analysis: an in vitro assessment

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Abstract The prediction of tumor resistance to antineoplastic drugs remains an important challenge in cancer chemotherapy. Several methods have been proposed in this connection, but they present a number of problems such as clinical relevance and applicability. In the present work we put forward an original methodology to assess the drug sensitivity of cancer cells. For this purpose we submitted chemosensitive and chemoresistant cell lines to different anticancer drugs and monitored the cell growth and the drug-induced morphonuclear effects by means of digital cell-image analysis of Feulgen-stained nuclei. The results showed that drug-induced effects at the morphonuclear level correlated statistically with the effects produced at the cell proliferation level. For example, the mean nuclear size value increased as a function of the drugs' efficiency recorded at the cell proliferation level. In the same way, the frequency of large dense chromatin clumps also increased in accordance with the drugs' efficiency. The present work thus demonstrates that digital cell-image analysis can be applied to monitor the efficiency of chemotherapeutic treatment carried out on cell lines in vitro. The present methodology could possibly be used on solid tumors, from which biological material can be obtained serially by means of fine-needle aspiration. As evidence of this, the present methodology can also be applied to hematological cancers.

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Key words Drug response · Image cytometry · Feulgen staining

Introduction

The most important problem in chemotherapy as applied to most solid human cancers consists of tumor resistance to the treatment [14, 27, 48]. The possibility of predicting cancer response to chemotherapy thus remains an important challenge. Various methods have been proposed for the purpose of forecasting tumor response to antineoplastic drugs [9, 34, 44]. It seems that the most helpful method for the study of tumor response to antineoplastic drugs remains the in vitro culture of human tumors in semisolid media [9, 23]. Because of this, a lot of work is currently being focused on the clinical relevance of the expression of P-glycoprotein, a transmembrane protein produced in many resistant cancer cells [26]. Many studies describe a relationship between drug resistance and the expression of P-glycoprotein [2, 22, 27, 36, 43]. Nevertheless, other mechanisms contribute to drug resistance, e.g., the overexpression of methallothionein [28], altered topoisomerase II [35], or an increase in DNA repair [7].

In the present work, the effects induced at the morphonuclear level by a number of antineoplastic agents were correlated with those recorded at the cell proliferation level. Eight antineoplastic drugs and three sensitive versus three chemoresistant cell lines were included in the study. Morphonuclear characteristics were assessed by means of computer-assisted microscope analyses of Feulgen-stained nuclei. The eight drugs involved various mechanisms of action since they included two alkylating agents, i.e. melphalan (L-PAM) [11] and the investigational agent PE1001 [32]; two antimetabolites, i.e., 5-fluorouracil (5-FU) [21a] and hydroxyurea (HC) [15]; an intercalating agent, i.e., doxorubicin (DOX) [8] a glycopeptide antibiotic, i.e., bleomycin (BLEO) [16]; a semisynthetic alkaloid,

i.e., vinorelbine (VRB) [12]; and a podophyllotoxine derivative, i.e., etoposide (VP-16) [45].

Materials and methods

Drugs

L-PAM, 5-FU, and DOX came from Sigma Chemical Co. (USA); PE1001 and VRB, from Pierre Fabre Médicaments (France); HC, from Squibb (France); BLEO, from Laboratoire Roger Bellon (France); and VP-16, from Seripharm (France).

Cell culture

The six lines used were derived from the MXT, J82, and T24 cell lines. The MXT cell line [29] was derived from the MXT mouse mammary adenocarcinoma [47]. The J82 [37] and T24 [6] cell lines came from the American Type Culture Collection (HTB 1 and HTB 4, respectively); both stemmed from human bladder cancers. Two variants were set up for each cell line. The first involved a chemosensitive phenotype (labeled MXT/S, J82/S, and T24/S) and the second, a chemoresistant one (labeled MXT/R, J82/R, and T24/R). The description of how the chemosensitive and chemoresistant cell lines were obtained is given elsewhere [38].

The cells were maintained as monolayers as previously described elsewhere [31, 38]. 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% fetal calf serum, L-glutamine, and antibiotics (all the solutions came from Gibco, UK).

Preparation of the samples

The preparation of the samples was similar to that previously described elsewhere [38, 39]. Briefly, aliquots of 2.5 ml of a suspension containing 10,000 cells/ml in the exponential growth phase were plated in petri dishes (Nunc, Denmark) containing glass coverslips to which the cells could adhere and proliferate. Except in the cases of samples serving as controls (CT), the medium was supplemented after 24 h of incubation with 0.2 ml of a solution containing one of the eight antineoplastic agents used in this work. The final concentrations of the antineoplastic drugs were $10^{-8}M$ for VRB, $10^{-7}M$ for DOX, BLEO, and VP-16; $10^{-5}M$ for L-PAM, PE1001, and 5-FU; and $10^{-4}M$ for HC. These concentrations were chosen on the basis of previous results [17, 39, 42], so as to obtain a significant decrease in the cell proliferation of the sensitive lines, i.e., a proliferation of between 10% and 50% of that of the control cells.

At 72 h after addition of the drugs (24 h in the case of VRB-treated cells), the glass coverslips to which the cells had adhered were fixed in a mixture of ethanol (75 ml), 40% formol (20 ml), and 100% acetic acid (5 ml) and mounted on microscope slides by means of DPX (BDH Chemicals, UK). In the case of VRB, we studied the drug-induced morphonuclear effects after an incubation period of only 24 h because after 72 h there were no longer any cells in the mitotic phase (i.e., the phase where the VRB acts). The slides were then subjected to the Feulgen reaction after hydrolysis in HCl (6 *M*) for 1 h at 24°C as previously described [31]. These Feulgen-stained slides were used to study the antineoplastic drug-induced effects on cell proliferation and the morphonuclear features. Each experimental condition was carried out in triplicate for each of the six cell lines tested.

Cell growth assessment

The number of Feulgen-stained cell nuclei present on an area of 16 mm^2 was recorded for each slide analyzed. This assessment was carried out using a Dialux 20 EB microscope (Leitz, Germany; 40 × lens magnification) equipped with a 100-case grid. In all, 5 randomly determined areas were analyzed per slide, resulting in 15 areas per experimental condition. The values thus obtained permitted assessment of the cytotoxic effect of the drugs on the proliferation of the cell lines under the different experimental conditions tested. From an experimental point of view, it was observed that the number of control cells quadrupled within 72 h (the period of incubation of cells in the presence of the drugs). This time corresponds to two cell-doubling times. Hence, in the case of treated cells as compared with the respective control condition (100%), a "cell proliferation" level of 25% means that the quantity of cells was smaller than the number of cells plated at the beginning of the drug treatment, and vice-versa.

Image analysis

A total of 300 cell nuclei were analyzed for each slide by means of a SAMBA 2005 system (Alcatel-TITN, France), thus enabling each of the nuclei analyzed to be characterized according to 15 morphonuclear parameters [5]. The first parameter analyzed was geometric, i.e., the nuclear area (NA), which describes the nuclear size. The second parameter was the integrated optical density (IOD), which measures the DNA content [30]. The 13 other parameters describe the chromatin pattern. The mean optical density (MOD), the variance of the optical density (VOD), and the skewness (SK) and kurtosis (K) indices describe the chromatin texture at the densitometry level. The short- (SRL) and long (LRL)-run length emphases (which are representative of the frequency of small and large dense chromatin clumps, respectively), their distribution (RLD), their percentage (RLP), and the gray level distributions (GLD) involve parameters calculated on the basis of the length section matrices [20]. The remaining parameters were the local mean (LM), energy (E), the coefficient of variance (CV), and contrast (C), which were computed on the basis of the co-occurrence matrices [24]. The parameters have been described elsewhere [33, 38].

Statistical and mathematical analyses

The results of assessment of cell growth and the morphonuclear effects of the drugs are reported as mean values ± SEM. The means were statistically compared by means of the Fisher *F*-test (one-way analysis of variance). Equality of variance was checked by the Bartlett test. Correlations were assessed by means of the Kendall nonparametric test.

Results

Cell proliferation

Figure 1 illustrates the cell proliferation of the MXT, J82, and T24 sensitive (/S) and resistant (/R) cell lines after 72 h of incubation either without drugs (CT) or in the presence of the eight distinct antineoplastic agents. Each drug induced a highly significant decrease in the proliferation ($P < 0.01$ to $P < 0.001$) of the sensitive cell line. With respect to the resistant cell lines, it appears that PE1001, DOX, VRB, and VP-16 did not

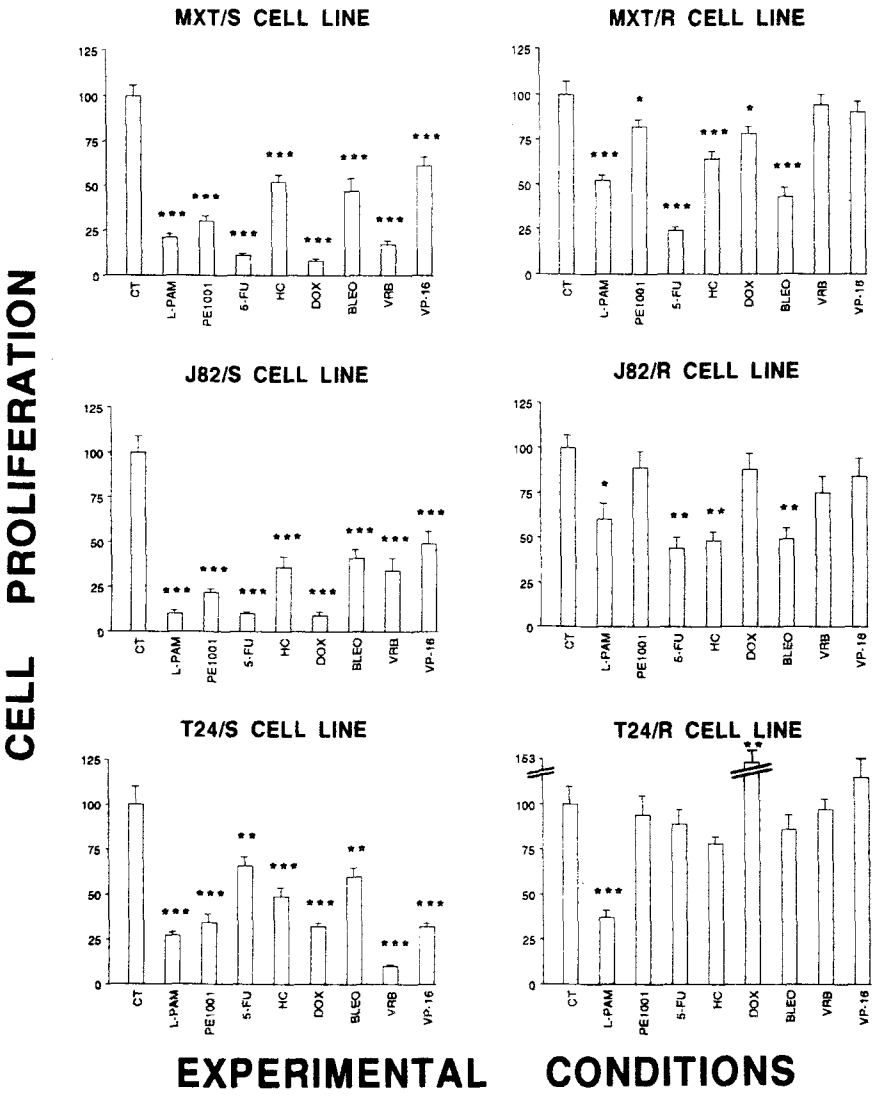

Fig. 1 Cell proliferation obtained in the six cell lines used in this work after 72 h incubation with the various drugs tested. The results are expressed in percentages of proliferation in relation to the control value ($\pm$ SEM). The level of significance was obtained by comparison with the controls ($*P < 0.05$; $**P < 0.01$; $***P < 0.001$). Statisticals tests were carried out using the Fisher *F*-test

induce any significant ($P > 0.05$) decrease in cell proliferation except in the case of the MXT/R line, where PE1001 and DOX slightly decreased ($P < 0.05$) cell proliferation. DOX induced a significant increase in the growth of the T24/R cells ($P < 0.01$), a feature that might appear curious at first but has previously been observed by Vichi and Tritton [46]. L-PAM induced a significant decrease in the cell proliferation of all resistant lines ($P < 0.05$ to $P < 0.001$). 5-FU, HC, and BLEO induced a significant decrease in the cell proliferation of the MXT/R and J82/R lines ($P < 0.001$ and $P < 0.01$ respectively) but not in that of the T24/R line ($P > 0.05$).

Image analysis

Figure 2 illustrates the drug-induced effects on the nuclear area (NA). All the antineoplastic agents under study induced a marked increase in this mean parametric value ($P < 0.05$ to $P < 0.001$, respectively) in each sensitive cell line. The exception was VRB, which induced a decrease in this parameter in the case of both the J82/S and the T24/S cells ($P < 0.001$, respectively). In the same way, the 5-FU also induced a slight decrease in this mean parametric value ($P < 0.05$). In the case of the resistant cell lines, VRB and VP-16 slightly decreased the mean NA value in MXT/R and T24/R cells but had no apparent effect on the J82/R variant. Similar features were also observed when MXT/R and J82/R cells were exposed to DOX, when MXT/R cells were exposed to PE1001, and when T24/R cells were exposed to 5-FU. The other experimental conditions led to a significant increase in the mean NA value ($P < 0.05$ to $P < 0.001$). The results also show that the extent of the drug-induced effects on the mean NA was weaker in the resistant lines than in the sensitive

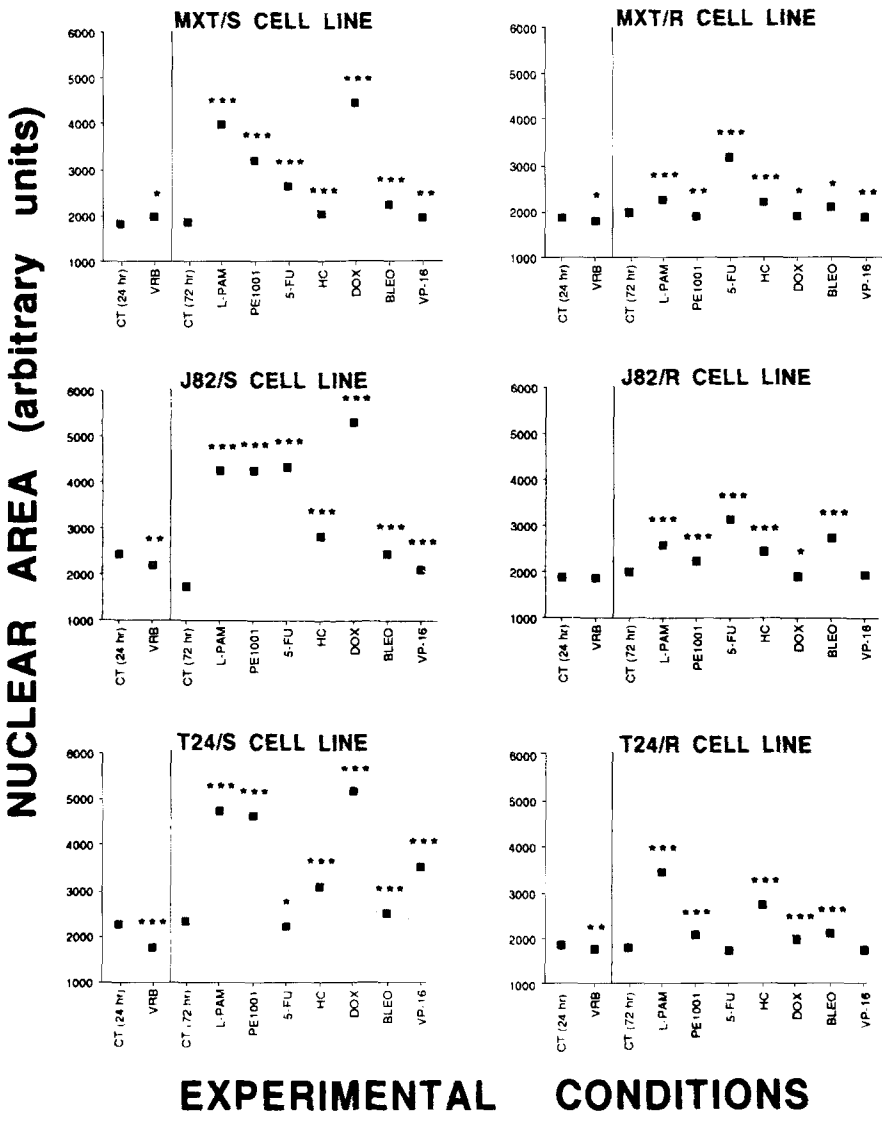

Fig. 2 Influence of the drugs on nuclear size (or nuclear area, NA). The results are expressed in pixels. Because SEM values are included in the *black squares*, they do not appear in the chart. The level of significance was obtained by comparison with the controls ($*P < 0.05$; $**P < 0.01$; $***P < 0.001$). Statisticals tests were carried out using the Fisher *F*-test

variants. Similar features were observed with respect to other parameters (data not shown).

Correlation between drug-induced effects on cell proliferation and those induced on the development of the morphonuclear parameters

As mentioned above, the results show that the drug-induced effects at the cell proliferation level were normally greater in the sensitive than in the resistant cell variants. The same feature was obtained with respect to the morphonuclear parameters.

Since drug-induced effects on cell proliferation seemed to develop in a manner similar to that of the effects on the morphonuclear parameters, we studied the correlations existing between these two kinds of value development. Because VRB led to anarchic modifications (Fig. 2), we calculated those correlations both by taking the VRB-induced effects into account and by ignoring them. Table 1 shows that the values obtained for most of the morphonuclear parameters under study were correlated either positively or negatively with the cell proliferation values. Each correlation was stronger when the VRB influence was eliminated than if it had not been removed from the statistical analysis. Table 1 shows that the NA for which the highest rank correlation coefficient was obtained was the best parameter for ascertaining drug-induced effects on the cells morphonuclear features in relation to cell growth. The observation nevertheless remains that other parameters such as LRL, RLP, LM, E, CV, and C also attained a rank coefficient that did not differ markedly from that attained by NA.

Table 1 Correlation between cell growth under the various experimental conditions tested in this work and the development of the parameters described by digital cell-image analysis as well as the Kendall correlation (r_k) calculated by means of the Kendall nonparametric test and the probability associated with these coefficients. Whereas the first column takes account of the eight drugs tested, the second does not take any account of VRB (*NS*, not significant)

	r_k		r_k	
NA	− 0.5481	(***)	− 0.6780	(***)
IOD	− 0.3251	(**)	− 0.3998	(**)
MOD	0.3760	(***)	0.5094	(***)
SK	− 0.2724	(**)	− 0.3482	(**)
VOD	0.1116	(NS)	0.2727	(**)
K	− 0.4602	(***)	− 0.5083	(***)
SRL	0.2621	(**)	0.3865	(**)
LRL	− 0.5262	(***)	− 0.6671	(***)
GLD	0.1024	(NS)	0.1362	(NS)
RLD	0.2537	(*)	0.3870	(*)
RLP	0.4847	(***)	0.6289	(***)
LM	− 0.5012	(***)	− 0.6577	(***)
E	− 0.5035	(***)	− 0.6540	(***)
CV	0.5165	(***)	0.6596	(***)
C	0.4915	(***)	0.6134	(***)

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ (NS = $P > 0.05$)

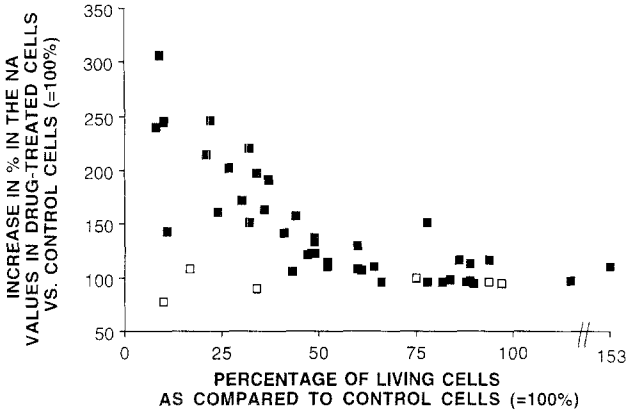

Fig. 3 Correlation existing between cell proliferation and the increase in NA under the various experimental conditions tested *white squares* VRB, *black squares* the seven other drugs)

In Figs. 3–5, we illustrate the correlation between the drug-induced effect on cell proliferation and 3 of the 15 parameters studied. The three parameters chosen were NA, IOD, and LRL (the "best" textural parameter in terms of correlation with cell proliferation; see Table 1). In Figs. 3–5, each experimental condition is represented by a square. The white squares represent the experimental conditions under which the six cell lines were treated with VRB, and the black squares represent all other experimental conditions, i.e., the effects induced by all the remaining drugs under study. Figures 3 and 5 clearly show that the antineoplastic agents (except VRB) led to an inverse relationship between an increase NA (Fig. 3) and LRL values (Fig. 5) and

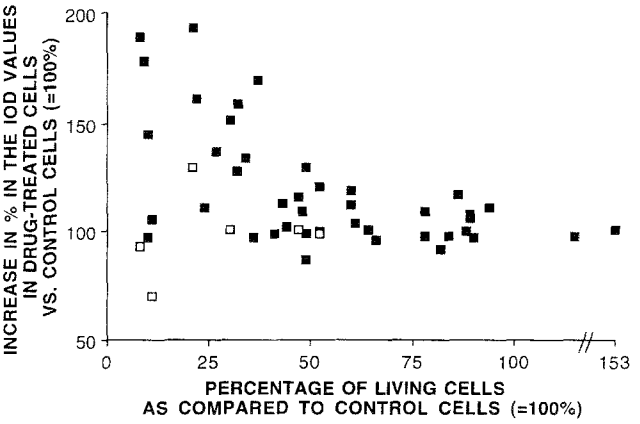

Fig. 4 Correlation existing between cell proliferation and the increase in DNA content under the various experimental conditions tested (*white squares* VRB, *black squares* the seven other drugs)

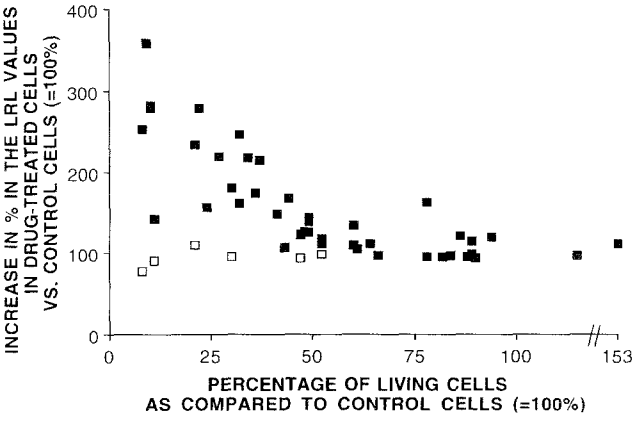

Fig. 5 Correlation existing between cell proliferation and the increase in LRL under the various experimental conditions tested (*white squares* VRB, *black squares* the seven other drugs)

a decrease in cell proliferation. In contrast, Fig. 4 shows that the correlation found between IOD and cell proliferation was poor as compared with the correlations obtained with NA and LRL.

Discussion

Several publications have previously demonstrated that many antineoplastic drugs induce marked morphonuclear modifications that correspond to an increase in nuclear size and DNA content [4, 10, 39]. In addition, Colomb and colleagues [10] report that no chromatin pattern change has been observed in anthracycline-resistant cells treated with daunorubicin. We accordingly subjected six cell lines (with different drug sensitivities) to eight antineoplastic agents with different mechanisms of action. The purpose of this experiment was to correlate the drug-induced effects on cell growth with the effects recorded for morphonuclear changes.

To study the drug-induced effects on cell growth, the number of cell nuclei stained with the Feulgen reagent were counted. It has previously been demonstrated that this method gives results similar to those obtained with the more conventional test based on the reduction of tetrazolium dye in formazan (MTT test) [17]. Nevertheless, in some cases the method used in the present work showed cytotoxic effects that were significantly more pronounced as compared with those observed for the MTT test. These differences were attributed to the distinct culture conditions of the two tests [17]. The sensitivity (and resistance) of the various cell lines used in the present work have been studied in a previous work by means of the MTT test [41]. The results obtained in that study were in accordance with the present work except in the case of BLEO, which was significantly more effective under the present experimental conditions as compared with the results obtained by means of the MTT test [41].

The morphonuclear-induced effects were monitored by means of digital cell-image analysis. Following the methodology used in the present work, the measurements were made on cells that remained attached to the coverslips. A possible inconvenience of this methodology lay in the observation that cell subpopulations were lost in the culture system, especially in cases where survival was low. However, at the end of the drug incubation period, the numbers of cells that were not fixed on the coverslips were low as demonstrated by a microscope estimation. Furthermore, it is possible that these cells were dead, necrotic, or apoptotic.

The results show that the chemotherapy induced morphonuclear modifications, the extent of which correlated significantly, either positively or negatively, with the decrease in proliferation. Of the 15 parameters under study, the most interesting seemed to be the nuclear area (NA), which is representative of nuclear size. Indeed, the drug-induced increase in the mean NA value had a highly negative correlation ($P < 0.001$, $r_k = -0.6780$) with the drug-induced decrease in cell proliferation (Table 1, Fig. 3). These results are corroborated by those of Briffod and colleagues [4], who report from a clinical study that the NA increases significantly in treated chemosensitive cells. The observed increase in nuclear size following chemotherapy is explained at least partly by the finding that the majority of the antineoplastic drugs used in the present study led to arrest of the cells in the S or G_2 phases of the cell cycle (data not shown). Indeed, the S and G_2 phases of the cell cycle are characterized by an increase in nuclear size. Among the various anticancer drugs used in the present experiments, only VRB was an exception to this rule; indeed, this vinca alkaloid led to an accumulation of cells in the M phase of the cell cycle, a cell-cycle phase characterized by small nuclei.

The present study clearly demonstrates that morphonuclear parameters quantitatively describing the chromatin pattern also develop in relation to antiproliferative drug efficiency. Hence, it appears that the development of various morphonuclear parameters can be used to monitor the efficiency of a given chemotherapy. One of these parameters, NA can easily be monitored without any sophisticated method or machine [1, 3, 19]. The present methodology may therefore be applied to clinical monitoring of antineoplastic drug efficiency in hematological and solid cancers, from which biological material can be serially obtained by means of fine-needle aspiration. It is thus possible to obtain cancer cells before and after treatment and, hence, to study the development of the morphonuclear features of cell nuclei under the effect of chemotherapy. If the results obtained in vitro in the present work are clinically relevant, it may be possible to ascertain rapidly the efficiency of the treatment and, if necessary, to modify it. This methodology has previously been used to monitor in vivo the chemotherapy- and radiotherapy-induced effects on the MXT mouse mammary model [21, 40]. Furthermore, this methodology can also make it possible to study the efficiency of resistance modifiers. Indeed, in chemoresistant cell lines, the well-known resistance modifier verapamil restores the morphonuclear features observed in DOX-treated chemosensitive cells [18].

It is noteworthy that the sensitive cell lines used in the present work display similar sensitivities to anticancer drugs and that the resistant ones possess a similar cross-resistance pattern [41]. The similarity seen between the different cross-resistance patterns is explained by the observation that the principal resistance mechanism brought into play by the resistant cell lines was due to expression of the multidrug resistance conferred by P-glycoprotein [13]. Therefore, these experiments will have to be reproduced in studies including a large series of cell lines with clearly differential sensitivities to specific drugs and possessing different resistance mechanisms. Indeed, as the resistant cell lines used in the present work possess the same resistance mechanism (i.e., the mechanism generated by P-glycoprotein), it is possible that the results of the present work were affected by this resistance mechanism. In addition, it will also be necessary to use other antineoplastic agents and a large series of drug concentrations. Indeed, with regard to the lack of accuracy in chemosensitivity prediction achieved with all test systems thus far available, it is too early to postulate on the basis of a single experiment that the measurement of the nuclear area makes it possible to discriminate easily between sensitive and resistant tumors. Furthermore, the drug-induced increase in the nuclear size of sensitive cells is not a universal effect. Indeed, VRB had different morphonuclear effects in comparison with the seven other drugs (Figs. 3–5). The observation nevertheless remains that monitoring of the efficiency of this pharmacological class of drugs (i.e., vinca-alkaloid derivatives) is possible by means of the quantitation of mitotic cells [25].

In conclusion, if the present data are clinically relevant, the present work strongly suggests that the efficiency of a given chemotherapy may be evaluated precociously by means of morphonuclear parameter assessment. Nuclear size–one of these parameters–is easily measurable, and its mean value develops as an inverse function of a drug's antiproliferative efficiency. The present method may be applied to both hematological and solid cancers, for which cytopunctures can be carried out serially. We are now pursuing our experiments so as to validate the present methodology clinically. We are also setting up a model to describe the chromatin pattern of the chemoresistant cells that are initially present in any human cancer and can be responsible for the failure of treatment, i.e., for regrowth of the tumor during or after chemotherapy.

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