Decrease in c-Myc activity enhances cancer cell sensitivity to vinblastine

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The c-*myc* oncogene encodes for a transcriptional factor involved in many cellular processes such as proliferation, differentiation and apoptosis. According to these different functions, the role of c-Myc protein in cellular sensitivity to anti-cancer drugs is controversial. We defined the role of c-Myc in cancer cell sensitivity to vinblastine (VLB) using human colon cancer cells: LoVo wild-type or transfected with a plasmid containing the human c-*myc* gene in antisense orientation (LoVo-mycANS). Analysis of VLB cytotoxicity demonstrated a 3-fold increase in VLB sensitivity in LoVo-mycANS cells. Comparison between cells revealed different apoptosis kinetics: accumulation of cells in sub-G_1 phase and poly(ADP-ribose) polymerase cleavage occurred earlier in LoVo-mycANS. Then, we demonstrated a mitochondrial membrane potential disruption followed by cytochrome *c* release that indicates the involvement of mitochondria in this apoptotic signaling pathway. This earlier apoptosis was accompanied by a Bcl-2 decrease and a p53 increase. In conclusion, the decrease in c-Myc expression enhanced the VLB sensitivity, triggering earlier apoptosis through induction of the intrinsic pathway. Thus, c-*myc* induction is a resistance factor and our findings suggest that tumors carrying low levels of c-Myc protein could be more responsive to vinca alkaloids treatment. Moreover, the downregulation of c-*myc* oncogene by an antisense strategy might represent a useful goal for improving the efficacy of this anti-neoplastic drug family. *Anti-Cancer Drugs* 17:181–187

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Introduction

Maintenance of normal function in cells depends on a precise regulation of multiple signaling pathways that control cellular decisions to either proliferate, differentiate or initiate apoptotic cell death. Deregulation of the balance between these distinct processes leads to human diseases such as cancers.

The c-*myc* protooncogene encodes for the c-Myc transcription factor, which plays an important role in cellular proliferation. The increase of c-Myc in response to various mitogenic stimuli is required for cells to enter into the S phase [1]. Concomitantly with promoting cell proliferation, c-Myc inhibits terminal differentiation and sensitizes cells to apoptosis [2]. In fact, c-*myc* is induced by a variety of apoptotic stimuli, including cytokines, hypoxia, DNA damage and chemotherapeutic agents [3]. Despite intensive research, the molecular mechanisms involving c-*myc*-mediated apoptosis are not as yet understood [4]. Its overexpression in a large subset of tumors [5] suggests that this oncogene represents an attractive target for cancer therapy.

Although c-*myc* has been involved in both proliferation and cell death, the role of c-Myc protein in cellular susceptibility to anti-cancer drugs is controversial [6]. Indeed, its overexpression has been reported to increase chemotherapeutic sensitivity to adriamycin, camptothecin and etoposide [7,8], whereas it induces resistance to cisplatin [9]. Microtubule-damaging agents (MDAs) are clinically used as anti-cancer drugs for the treatment of various types of tumors. Some of these compounds inhibit microtubule polymerization (e.g. vinca alkaloids), whereas others stabilize microtubules (e.g. taxanes). Relationships between c-*myc* expression in tumor cells (over/down expression) and response to MDA treatment have not as yet been precisely established. Bottone *et al.* [10] have shown that high c-*myc* amplification potentiates the cytotoxic and apoptogenic effect of paclitaxel, whereas Knapp *et al.* [11] have shown a dramatic decrease in tumor growth after paclitaxel preceded by c-*myc* antisense oligonucleotide treatment. Moreover, to our knowledge, no data concerning the response to microtubule depolymerizing agents [i.e. vinca alkaloids including vinblastine (VLB)] and c-*myc* expression have yet been reported.

MDAs disrupt normal microtubule function by suppressing their dynamics, thus disturbing cell cycle progression usually leading to a mitotic arrest [12]. Additionally, their

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efficiency has largely been related to apoptosis induction [13]. We have previously shown that MDAs induce apoptosis through intrinsic signal pathway activation [12,14]. This mitochondrial pathway is initiated by permeabilization of the outer mitochondrial membrane associated with mitochondrial membrane potential ($\Delta\Psi_m$) disruption and followed by release of apoptogenic factors such as cytochrome *c*, which lead to apoptosome formation and caspase cascade activation. The mitochondrial membrane permeabilization might result from a direct effect of MDAs [15–17]. Moreover, members of the Bcl-2 family are central regulators of the intrinsic pathway [18]. Bcl-2 protein in particular is an anti-apoptotic mitochondrial membrane-associated protein that prevents the release of cytochrome *c* and caspase activation. It has been reported that MDA treatment leads to Bcl-2 phosphorylation and decreases its expression [19]. In addition, p53, normally induced and activated in response to DNA damage, is induced by MDA treatment [20]. The wild-type p53 tumor suppressor gene encodes a DNA-binding transcription factor that is able to mediate cell cycle checkpoint and induction of apoptotic cell death [21]. c-Myc and its partner Max have a binding site on the p53 promoter, suggesting that p53 is a target of c-Myc [22]. The role of p53 status on cell sensitivity to MDAs is controversial [23].

Our previous data showed that c-*myc* expression is induced by MDAs such as VLB [24]. Subsequently, we investigated the mechanism by which c-*myc* influences this sensitivity using a human colon carcinoma cell line and clones harboring different levels of c-Myc: LoVo parental cells [(LoVo-wild-type (W) and LoVo-mycANS (low c-*myc* level)].

In this study, we demonstrated that inhibition of c-*myc* in LoVo-mycANS leads to an increase in cell sensitivity to VLB treatment. This enhanced sensitivity is due to an apoptosis induction that occurs earlier and for lower VLB concentrations than those in LoVo-W. We also demonstrated that an intrinsic mitochondria apoptotic pathway is involved whatever the c-*myc* status, and that modifications affected the anti-apoptotic protein Bcl-2 and the tumor suppressor p53 expression in LoVo-mycANS.

Materials and methods

Cell lines and reagents

LoVo human colon adenocarcinoma cells (LoVo-W, and LoVo-mycANS-9 and -10), previously characterized (*myc* expression level and activity [25]), were maintained in culture as previously described [25]. LoVo-W were stably transfected using a vector carrying the c-*myc* sequence in the antisense orientation (LoVo-mycANS-9 and -10). LoVo-control cells were stably transfected using the empty vector $pCDNA_3$.

Cells were seeded 24 h before treatment at 2.5×10^4, 2×10^4 and 3.3×10^4 cells/cm^2 for LoVo-W, LoVo-control and LoVo-mycANS cells, respectively, depending on their different growth rate.

Stock solution of VLB (Lilly, Saint Cloud, France) was prepared in distilled water (10^{-3} mol/l) and kept frozen.

c-Myc transactivity

c-Myc transactivity was measured by transient transfection of either the M4-min-CAT (chloramphenicol acetyl transferase) or the min-CAT vector. The M4-min-CAT vector was a kind gift from R. Eisenman (Fred Hutchinson Cancer Research Center, Seattle, Washington, USA). It contains a four-tandem repeat of the consensus c-*myc* binding site upstream a minimal thymidine kinase promoter controlling the expression of the CAT gene. The min-CAT vector is identical to M4-min-CAT, but lacks the c-*myc*-binding sites. Cells were transfected using Xtreme gene Q2 (Roche, Mannheim, Germany). The relative levels of CAT activity in each cell line obtained from the M4-min-CAT were adjusted for background levels using the data from the min-CAT transfection [25].

Cytotoxicity assay

VLB cytotoxicity on LoVo cells was assessed after 72 h of treatment by the tetrazolium assay (MTT) as previously described [26]. The IC_{50} values were then determined as the concentration of VLB required to reduce the cell number by 50%.

[^{3}H]VLB cellular accumulation

To evaluate the intracellular VLB accumulation, cells were incubated with 1 or 3 nmol/l [^{3}H]VLB (10.8 Ci/mmol; Amersham Biosciences, Freiburg, Germany) for 6 h. Then, they were harvested, washed 4 times in PBS and lysed for 30 min at 4°C in RIPA buffer (20 mmol/l Tris–HCl, pH 8.0, 200 mmol/l NaCl, 1% Triton, 1 mmol/l EDTA and protease inhibitors). Total protein amount of each cellular lysate was determined using the Bio-Rad Protein Assay Reagent (Bio-Rad, Munich, Germany). Cell lysates were counted for radioactivity by liquid scintillation (LS1707; Beckman, Fullerton, California, USA) [15].

Cell cycle analysis by flow cytometry

After drug treatment, cells were harvested, fixed in methanol (70%, 20 min at −20°C), washed 4 times with PBS and stained with propidium iodide (20 µg/ml) for 30 min at room temperature. DNA content was measured by flow cytometry (FACSort; Becton Dickinson, Mississauga, Canada), and the percentage of cells in the G_0/G_1, S and G_2/M phases was determined (CellQuest software) [26].

Western blotting

After treatment, cells were washed with PBS and lysed for 30 min at 4°C in RIPA buffer. Cell lysates were

subjected to SDS–PAGE and transferred to nitrocellulose membrane. Membranes were then probed with either an anti-poly(ADP-ribose) polymerase (PARP) mouse monoclonal antibody (Zymed, San Francisco, California, USA) (1:500), an anti-p53 mouse monoclonal antibody (Dako, Glostrup, Denmark) (1:100), an anti-Bcl-2 mouse monoclonal antibody (Dako) (1:100) or with an anti-$p21^{WAF1}$ mouse monoclonal antibody (Oncogene, Darmstadt, Germany) (1:100). Then, blots were labeled with a peroxidase-conjugated secondary antibody (Jackson ImmunoResearch, Baltimore, Maryland, USA) (1:2000). Visualization was performed by chemoluminescence. The equal loading was confirmed by Ponceau S solution staining (Fluka, Steinheim, Germany).

Microscopy fluorescence studies: DAPI staining and cytochrome *c* visualization

Cells were grown on eight-well plates (Labtek, USA), and incubated with 3 nmol/l VLB for 24 and 48 h. They were then fixed with 3.7% formaldehyde, permeabilized with 0.1% saponine, and incubated with the anti-cytochrome *c* antibody (PharMingen, San Diego, California, USA) and secondary antibody coupled with FITC (Amersham) as previously described [17]. An additional incubation with DAPI (Sigma, Steinheim, Germany) was realized to stain nuclei [27]. Cells were observed using a Leica DM-IRBE microscope coupled with a digital camera (CCD camera coolsnapFX; Princeton Instruments, Trenta, New Jersey, USA). Four hundred cells were analyzed with Metamorph software.

Cytofluorimetric analysis of $\Delta\Psi_m$

For analysis of $\Delta\Psi_m$, cells were incubated with 3 nmol/l VLB for 24, 48 or 72 h. Then, they were harvested, incubated with 100 nmol/l of 3,3′-dihexyloxacarbocyanin iodide (DiOC6) (Molecular Probes, Leiden, Netherlands) for 30 min and analyzed by flow cytometry as previously described [14]. To ensure that DiOC6 uptake was specific for $\Delta\Psi_m$, we also treated cells with 50 µmol/l carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), which is a protonophore that dissipates the $\Delta\Psi_m$.

Statistical analysis

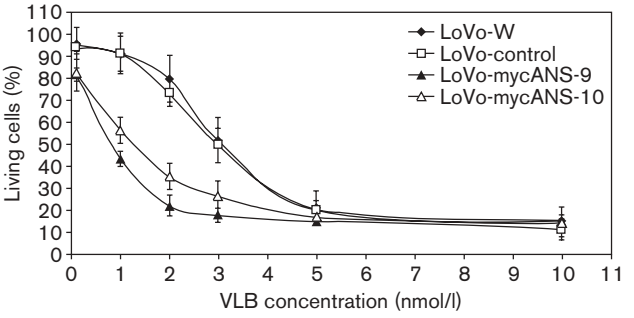
Each experiment was performed at least in triplicate. Statistical significance was performed with Student's *t*-test for comparison between the means; difference between two conditions was retained for $P < 0.05$.

Results

c-*myc* downregulation increases VLB sensitivity

First, we checked the transfection and RNA antisense efficiency in different clones (LoVo-mycANS-5, -9, -10 and -13) as previously described [25]. LoVo-mycANS-5 and -13 did not show a decreased *myc* activity, whereas LoVo-mycANS-10 showed a weak (–60%) reduced c-*myc* transactivity (data not shown). LoVo-mycANS-9 showed the most (–90%) reduced *myc* transactivity (as previously described [25]).

Fig. 1



c-myc downregulation sensitizes cells to VLB. Concentration dependence for cell growth in the presence of VLB in parental (LoVo-W), LoVo *myc* antisense (LoVo-mycANS) and LoVo-control cell lines. Cells were treated for 72 h with increasing concentrations of VLB and cell proliferation was assessed using the MTT reagent. LoVo-mycANS-9 and -10 showed statistically decreased IC_{50} compared with LoVo-W ($P < 0.05$).

To evaluate the role of c-*myc* on drug sensitivity, the effects of growing VLB concentrations on LoVo-W, LoVo-control, and LoVo-mycANS-9 and -10 were determined using the MTT assay. As shown in Fig. 1, VLB inhibited 50% of cell growth at 1 and 1.3 nmol/l in LoVo-mycANS-9 and -10, respectively, whereas 50% of cell growth inhibition required 3 nmol/l VLB for both LoVo-control and LoVo-W. LoVo-control cells showed the same VLB sensitivity as LoVo-W cells, indicating that the transfection process alone was not able to generate the gain in sensitivity observed in LoVo-mycANS-9 or -10. LoVo-mycANS-9 displayed a 3-fold higher sensitivity to VLB as compared with LoVo-W ($P < 0.05$). Therefore, we decided to compare LoVo-W to LoVo-mycANS-9 in further experiments.

Since VLB is a substrate of efflux pumps (like P-glycoprotein and MRP1), we evaluated the accumulation of [^{3}H]VLB in both cell lines. After 6 h of 3 nmol/l VLB treatment, we found no significant difference in LoVo-W and LoVo-mycANS-9 (data not shown). Therefore, the difference in sensitivity could not be explained by a difference in drug accumulation.

The increase in VLB sensitivity generated by c-*myc* downregulation is associated with an earlier apoptosis induction

Vinca alkaloids are known to induce mitotic block in proliferating cells by inhibition of microtubule dynamics and/or depolymerization of the microtubule network [12]. To evaluate whether the difference in sensitivity to VLB between LoVo-mycANS and LoVo-W was related to a difference in VLB-mediated cell cycle perturbations, we studied the cell cycle progression (data not shown). Three VLB concentrations were tested: 1 nmol/l (IC_{50} in

LoVo-mycANS-9), 3 nmol/l (IC_{50} in LoVo-W) and a high concentration of 30 nmol/l. We found the classical effects of the anti-mitotic agent VLB: a G_2/M phase accumulation (24 h treatment), later followed by a shift toward the sub-G_1 phase (48 h treatment) that indicates DNA fragmentation during apoptosis. However, some differences in extent of events are detectable.

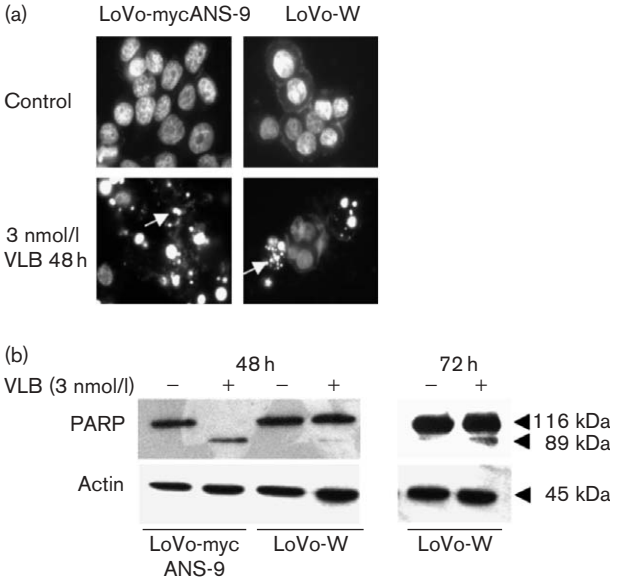
Indeed, 24 h treatment with 1 nmol/l VLB (IC_{50} value) blocked 40% of LoVo-mycANS-9 cells in the G_2/M phase, whereas 3 nmol/l VLB (IC_{50} value) led to a G_2/M phase blockage of 67% of LoVo-W. Thus, in LoVo-W, cell accumulation in the G_2/M phase is higher than in LoVo-mycANS-9 at respective IC_{50}s. This is consistent with the fact that LoVo-W grow faster [25]. In LoVo-mycANS-9, 30% of cells were already in the sub-G_1 phase after treatment with 3 nmol/l (versus 7% in untreated cells). Apoptosis induction rapidly occurs in parallel to G_2/M block induction.

After 48 h of treatment with 3 nmol/l VLB, accumulation of most cells in the sub-G_1 phase was already evident in LoVo-mycANS-9 (63 versus 7% in untreated cells). In LoVo-W, no significant difference was detectable between treated and untreated cells (13 versus 10%, respectively). It was only detectable after 72 h of VLB treatment (48%) in LoVo-W with the same concentration. Therefore, for the respective IC_{50}, apoptosis in LoVo-mycANS-9 seems to occur earlier than in LoVo-W. To explain this difference in sensitivity, we then studied the apoptotic pathway induced on both cell lines.

We first confirmed the apoptosis, and its earliness in LoVo-mycANS-9, by visualizing the chromatin condensation and nuclear fragmentation (Fig. 2a). After 48 h of 3 nmol/l VLB treatment, we showed that the percentage of apoptotic cells was 3-fold higher ($P < 0.05$) in LoVo-mycANS-9 than in LoVo-W (15 ± 2 and $5 \pm 1.5\%$, respectively). This result is consistent with the enhanced accumulation of sub-G_1 cells observed in LoVo-mycANS-9 after 48 h of VLB treatment. We then studied the proteolytic PARP cleavage in VLB-treated LoVo-W and LoVo-mycANS-9 cell lines. A specific cleavage of the 116-kDa PARP to a 89-kDa proteolytic fragment occurs during the apoptosis process concomitantly with final events [28]. As shown in Fig. 2(b), the PARP cleavage was total as early as 48 h of treatment with 3 nmol/l VLB in LoVo-mycANS-9, whereas it began at 72 h in LoVo-W. Thus, after 3 nmol/l VLB treatment, LoVo-mycANS cells started an earlier apoptosis.

Together, all these data indicate that c-*myc* downregulation increases VLB sensitivity in a manner closely related to an earlier induction of apoptosis. We then investigated whether apoptosis shown in LoVo-W and in LoVo-mycANS-9 followed the same signaling pathway.

Fig. 2



The increase in VLB sensitivity generated by c-*myc* downregulation is associated with an earlier apoptosis induction. (a) DAPI staining of cells visualized by fluorescent microscopy. Apoptotic cells contain fragmented nuclei (arrow). LoVo-W and LoVo-mycANS-9 cells were incubated with 0 (control) and 3 nmol/l VLB for 48 h. (b) Immunoblotting analysis of PARP cleavage from 116 to 89 kDa. LoVo-W and LoVo-mycANS-9 were incubated with 0 (control) and 3 nmol/l VLB for 48 and/or 72 h. Actin immunoblot serves as a control.

Increased VLB sensitivity is associated with an earlier loss of $\Delta\Psi_m$ and an earlier cytochrome *c* release

$\Delta\Psi_m$ disruption is usually associated with the opening of mitochondrial channels and with the subsequent release of apoptotic factors such as cytochrome *c* [29]. In order to investigate the potential involvement of mitochondria in VLB-induced apoptosis, we evaluated $\Delta\Psi_m$ variations by DiOC6 incorporation and flow cytometry analysis. We quantified the percentage of depolarized cells, i.e. cells with loss of $\Delta\Psi_m$, after treatment with 3 nmol/l VLB. The percentage of depolarized cells significantly ($P < 0.05$) increased in a time-dependent manner in both cell lines (15.1 ± 1.0, 22.1 ± 0.6 and $33.2 \pm 2.8\%$ in LoVo-W and 19.8 ± 3.9, 30.4 ± 4.2 and $35.4 \pm 1.4\%$ in LoVo-mycANS-9 after a 24, 48 and 72 h of treatment, respectively). Thus, mitochondria are affected in both cell lines under VLB treatment. Interestingly, as shown in Fig. 3, after 24 h of treatment, the percentage of depolarized cells is more important in LoVo-mycANS-9 than in LoVo-W as compared with control (2- and 1.6-fold, respectively). These data suggests that the intrinsic pathway may be activated earlier in LoVo-*myc*-ANS-9.

The data was confirmed by cytochrome *c* immunofluorescence analysis (data not shown). After 24 h of treatment with 3 nmol/l VLB, cytochrome *c* was released in the

cytosol of LoVo-mycANS-9 and remained in the mitochondria of LoVo-W cells. It was detectable in the cytosol only after 48 h in LoVo-W.

Thus, the mitochondrial pathway was involved in both cell lines and the increased sensitivity was accompanied by its earlier activation in LoVo-mycANS-9.

Apoptosis induction in LoVo-mycANS is associated with Bcl-2 downregulation

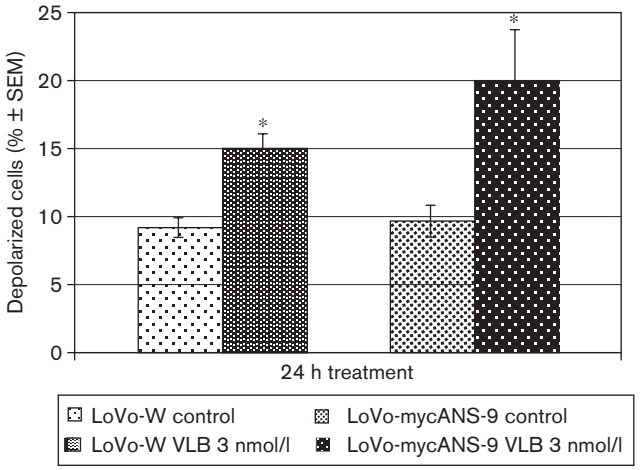
To further investigate the signaling pathway involved in mitochondrial VLB-induced apoptosis, we determined the expression level of the anti-apoptotic protein Bcl-2. Western blot was performed with total extracts of cell treated from 24 to 72 h with 3 nmol/l VLB (Fig. 4).

The basic level in the two cell lines was different: in LoVo-mycANS-9, Bcl-2 was highly expressed; in LoVo-W, it was undetectable. After 24 h of VLB treatment, no modification was observed. After 48 h of VLB treatment, we noticed a decrease in Bcl-2 level in LoVo-mycANS-9. This decrease was maintained after 72 h of treatment, whereas its level remained undetectable in LoVo-W.

As Bcl-2 can be downregulated by the tumor suppressor p53, we analyzed the p53 protein content and also found differences between cell lines. In LoVo-mycANS-9, the basal level of p53 was low, whereas this protein was strongly expressed in LoVo-W. After 3 nmol/l VLB treatment, no appreciable change was observed in LoVo-W even when we loaded a lower amount of protein in gels (data not shown). Furthermore, we noticed an induction of p53 expression in LoVo-mycANS-9 at 48 h of treatment, increasing with time.

Thus, the signal pathway leading to apoptosis modulates Bcl-2 and p53 expression in LoVo-mycANS-9. The involvement of Bcl-2 confirms the mitochondrial pathway induction.

Fig. 3

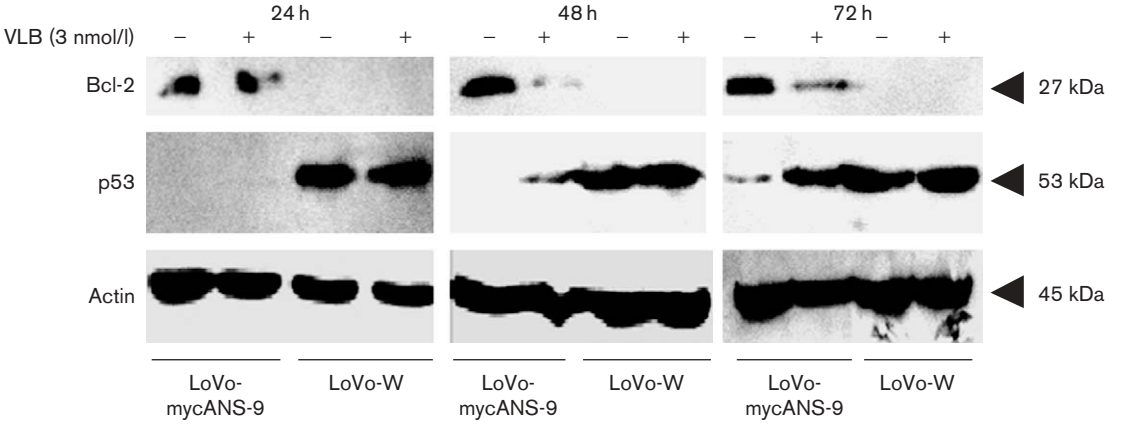


The increase in VLB sensitivity is associated with a higher $\Delta\Psi_m$ disruption in LoVo-mycANS-9 cells. $\Delta\Psi_m$ was assessed by DiOC6 staining followed by flow cytometry analysis. An increase in percentage of depolarized cells at 24 h (with disrupted $\Delta\Psi_m$) is noticed.

Discussion

We previously showed that VLB induced c-*myc* expression in different human cell lines [24,30], including those used in the present study (personal data). According to the paradoxical role of the c-*myc* oncogene in proliferation and apoptosis, this induction could be a mechanism of resistance or, on the contrary, a factor favoring cell

Fig. 4



VLB treatment modulates Bcl-2 and p53 expression in LoVo-mycANS-9. Bcl-2 and p53 expression was assessed by Western blotting in the LoVo-W and the LoVo-mycANS-9 cells after 24, 48 and 72 h of treatment with 0 (control) or 3 nmol/l VLB. Actin immunoblot serves as a control.

sensitivity to drug treatment. To define the exact role of c-*myc*, we investigated whether the effects of VLB could be modulated by the c-*myc* activity level. We found that downregulation of c-*myc* increases VLB cell sensitivity associated with an apoptosis induction. This study is consistent with works showing that c-*myc* antisense oligonucleotide treatment increases susceptibility to cisplatin by activating apoptosis [6,31,32]. Our results are of interest as antisense RNA technology is more applicable for *in vivo* studies than an oligonucleotide approach. Moreover, they constitute a first approach to evaluate the relationships between c-*myc* expression and response to vinca alkaloids. This study supports the hypothesis that c-*myc* acts as a survival factor. Consequently, c-*myc* induction under VLB treatment [24,30] can be considered as a specific form of resistance that could delayed VLB-induced cell death.

As the less-sensitive LoVo-W cells grow faster and have a higher G_2/M block than LoVo-mycANS cells, the difference in VLB cytotoxicity can be neither related to the cell growth rate nor to the disturbances in cell cycle progression.

Interestingly, an interaction between c-Myc protein and tubulin has been described [33,34], and it is suggested that microtubules might act as a cytoplasmic reservoir for c-Myc protein [33,35]. One may hypothesize that the depolymerizing agent VLB, by disturbing Myc/tubulin interaction, releases c-Myc from microtubules. Myc could then act as an anti-apoptotic factor, inducing cell proliferation. In LoVo-mycANS, the level of c-*myc* released by VLB is probably too low to prevent apoptosis. In agreement with this hypothesis, personal data showed an increased sensitivity of LoVo-mycANS to the newest vinca alkaloid vinflunine. Other studies are required to determine whether similar variations occur with other pharmaceutical anti-cancer drug classes.

Otherwise, modulations in VLB-induced apoptotic pathways, depending on the c-*myc* level, could explain this difference of sensibility. Indeed, apoptosis occurs earlier in LoVo-mycANS-9 cells than in LoVo-W. Do these results indicate a faster cell death process in LoVo-mycANS-9 or do they reflect two distinct signaling pathways according to c-*myc* activity level?

Mitochondria play a key role in most apoptotic pathways by releasing several pro-apoptotic proteins such as cytochrome *c*. Our results suggest that, in both cell lines, apoptotic signals downstream of mitochondria share a cytochrome *c*-dependent common route. Then, what happens upstream of mitochondria?

Interestingly, p53 and Bcl-2 basal expression levels are inversely correlated. p53 has been described to influence the expression of Bcl-2. First, the *bcl-2* gene has a p53 negative responsive element in its promoter [36]. Second, p53 can repress the transcription of Bcl-2 through the agency of one of its targets, e.g. Puma [2]. In addition, the low basal level of p53 protein in LoVo-mycANS-9 is explained by the fact that p53 is a target of c-Myc [37]. Lastly, it has also been reported that Myc can directly suppress Bcl-2 expression [38]. Thus, relationships between these three proteins are closed, and modulations of c-*myc* activity are likely to be responsible for Bcl-2 and p53 variations. After treatment, we showed a decrease in Bcl-2 expression level in LoVo-mycANS-9. These data are consistent with the fact that MDAs usually induce Bcl-2 downregulation leading to mitochondrial dysfunction and release of pro-apoptotic factors [19]. In parallel, we observed an increase in p53 expression level which was confirmed by p21 expression (data not shown). Interestingly, p53 has been shown to function as a pro-apoptotic factor [21]. Thus, p53 and Bcl-2 may, respectively, serve as an effector and repressor of a common cell death pathway [39]. The Bcl-2 decrease and p53 increase observed in LoVo-mycANS-9 after VLB treatment could facilitate apoptosis in this cell line.

In conclusion, c-*myc* downregulation increases cellular sensitivity to VLB. This sensitivity gain in low c-*myc* activity cells is associated with an earlier apoptosis that involves the mitochondrial signaling pathway. Thus, the enhancement of c-*myc* expression generated by VLB treatment is likely to constitute a specific form of resistance of cancer cells. In this way, tumors carrying low levels of c-Myc protein could be more responsive to VLB treatment. The downregulation of this oncogene with antisense strategy might represent a way of improving the efficacy of MDAs.

Acknowledgments

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References

1 Amati B, Alevizopoulos K, Vlach J. Myc and the cell cycle. *Front Biosci* 1998; **3**:d250–d268.
2 Nilsson JA, Cleveland JL. Myc pathways provoking cell suicide and cancer. *Oncogene* 2003; **22**:9007–9021.
3 Prendergast GC. Mechanisms of apoptosis by c-Myc. *Oncogene* 1999; **18**:2967–2987.
4 Biroccio A, Benassi B, Filomeni G, Amodei S, Marchini S, Chiorino G, *et al.* Glutathione influences c-Myc-induced apoptosis in M14 human melanoma cells. *J Biol Chem* 2002; **277**:43763–43770.
5 Nesbit CE, Tersak JM, Prochownik EV. MYC oncogenes and human neoplastic disease. *Oncogene* 1999; **18**:3004–3016.
6 Biroccio A, Benassi B, Fiorentino F, Zupi G. Glutathione depletion induced by c-Myc downregulation triggers apoptosis on treatment with alkylating agents. *Neoplasia* 2004; **6**:195–206.
7 Nesbit CE, Fan S, Zhang H, Prochownik EV. Distinct apoptotic responses imparted by c-myc and max. *Blood* 1998; **92**:1003–1010.
8 Arango D, Mariadason JM, Wilson AJ, Yang W, Corner GA, Nicholas C, *et al.* c-Myc overexpression sensitises colon cancer cells to camptothecin-induced apoptosis. *Br J Cancer* 2003; **89**:1757–1765.

9 Kinashi Y, Akaboshi M, Masunaga S, Ono K, Watanabe M. Resistance to 195mPt-radiolabeled *cis*-diaminedichloroplatinum (II) of SHOK cells transfected with various oncogenes. *Radiat Med* 1998; **16**:233–237.

10 Bottone MG, Soldani C, Tognon G, Gorrini C, Lazze MC, Brison O, *et al.*. Multiple effects of paclitaxel are modulated by a high c-*myc* amplification level. *Exp Cell Res* 2003; **290**:49–59.

11 Knapp DC, Mata JE, Reddy MT, Devi GR, Iversen PL. Resistance to chemotherapeutic drugs overcome by c-Myc inhibition in a Lewis lung carcinoma murine model. *Anticancer Drugs* 2003; **14**:39–47.

12 Pourroy B, Carre M, Honore S, Bourgarel-Rey V, Kruczynski A, Briand C, *et al.* Low concentrations of vinflunine induce apoptosis in human SK-N-SH neuroblastoma cells through a postmitotic G_1 arrest and a mitochondrial pathway. *Mol Pharmacol* 2004; **66**:580–591.

13 Jordan MA. Mechanism of action of antitumor drugs that interact with microtubules and tubulin. *Curr Med Chem Anticancer Agents* 2002; **2**:1–17.

14 Goncalves A, Braguer D, Carles G, Andre N, Prevot C, Briand C. Caspase-8 activation independent of CD95/CD95-L interaction during paclitaxel-induced apoptosis in human colon cancer cells (HT29-D4). *Biochem Pharmacol* 2000; **60**:1579–2584.

15 Carre M, Andre N, Carles G, Borghi H, Brichese L, Briand C, *et al.* Tubulin is an inherent component of mitochondrial membranes that interacts with the voltage-dependent anion channel. *J Biol Chem* 2002; **277**:33664–33669.

16 Andre N, Braguer D, Brasseur G, Goncalves A, Lemesle-Meunier D, Guise S, *et al.* Paclitaxel induces release of cytochrome *c* from mitochondria isolated from human neuroblastoma cells. *Cancer Res* 2000; **60**: 5349–5353.

17 Andre N, Carre M, Brasseur G, Pourroy B, Kovacic H, Briand C, *et al.* Paclitaxel targets mitochondria upstream of caspase activation in intact human neuroblastoma cells. *FEBS Lett* 2002; **532**:256–260.

18 Borner C. The Bcl-2 protein family: sensors and checkpoints for life-or-death decisions. *Mol Immunol* 2003; **39**:615–647.

19 Ganansia-Leymarie V, Bischoff P, Bergerat JP, Holl V. Signal transduction pathways of taxanes-induced apoptosis. *Curr Med Chem Anticancer Agents* 2003; **3**:291–306.

20 Wang LG, Liu XM, Kreis W, Budman DR. The effect of antimicrotubule agents on signal transduction pathways of apoptosis: a review. *Cancer Chemother Pharmacol* 1999; **44**:355–361.

21 Haupt S, Berger M, Goldberg Z, Haupt Y. Apoptosis - the p53 network. *J Cell Sci* 2003; **116**:4077–4085.

22 Roy B, Beamon J, Balint E, Reisman D. Transactivation of the human p53 tumor suppressor gene by c-Myc/Max contributes to elevated mutant p53 expression in some tumors. *Mol Cell Biol* 1994; **14**:7805–7815.

23 Abal M, Andreu JM, Barasoain I. Taxanes: microtubule and centrosome targets, and cell cycle dependent mechanisms of action. *Curr Cancer Drug Targets* 2003; **3**:193–203.

24 Bourgarel-Rey V, El Khyari S, Rimet O, Bordas B, Guigal N, Braguer D, *et al.* Opposite effects of antimicrotubule agents on c-*myc* oncogene expression depending on the cell lines used. *Eur J Cancer* 2000; **36**: 1043–1049.

25 Arango D, Corner GA, Wadler S, Catalano PJ, Augenlicht LH. c-myc/p53 interaction determines sensitivity of human colon carcinoma cells to 5-fluorouracil *in vitro* and *in vivo*. *Cancer Res* 2001; **61**:4910–4915.

26 Carles G, Braguer D, Sabeur G, Briand C. The effect of combining antitubulin agents on differentiated and undifferentiated human colon cancer cells. *Anticancer Drugs* 1998; **9**:209–221.

27 Garcia P, Braguer D, Carles G, Briand C. Simultaneous combination of microtubule depolymerizing and stabilizing agents acts at low doses. *Anticancer Drugs* 1995; **6**:533–544.

28 Darmon AJ, Nicholson DW, Bleackley RC. Activation of the apoptotic protease CPP32 by cytotoxic T-cell-derived granzyme B. *Nature* 1995; **377**:446–448.

29 Scarlett JL, Sheard PW, Hughes G, Ledgerwood EC, Ku HH, Murphy MP. Changes in mitochondrial membrane potential during staurosporine-induced apoptosis in Jurkat cells. *FEBS Lett* 2000; **475**:267–272.

30 Bourgarel-Rey V, Vallee S, Rimet O, Champion S, Braguer D, Desobry A, *et al.* Involvement of nuclear factor kappaB in c-Myc induction by tubulin polymerization inhibitors. *Mol Pharmacol* 2001; **59**:1165–1170.

31 Citro G, D'Agnano I, Leonetti C, Perini R, Bucci B, Zon G, *et al.* c-myc antisense oligodeoxynucleotides enhance the efficacy of cisplatin in melanoma chemotherapy *in vitro* and in nude mice. *Cancer Res* 1998; **58**:283–289.

32 Leonetti C, Biroccio A, Candiloro A, Citro G, Fornari C, Mottolese M, *et al.* Increase of cisplatin sensitivity by c-*myc* antisense oligodeoxynucleotides in a human metastatic melanoma inherently resistant to cisplatin. *Clin Cancer Res* 1999; **5**:2588–2595.

33 Alexandrova N, Niklinski J, Bliskovsky V, Otterson GA, Blake M, Kaye FJ, *et al.* The N-terminal domain of c-Myc associates with alpha-tubulin and microtubules *in vivo* and *in vitro*. *Mol Cell Biol* 1995; **15**:5188–5195.

34 El Khyari S, Bourgarel V, Barra Y, Braguer D, Briand C. Pretreatment by tubulin agents decreases C-MYC induction in human colon carcinoma cell line HT29-D4. *Biochem Biophys Res Commun* 1997; **231**: 751–754.

35 Niklinski J, Claassen G, Meyers C, Gregory MA, Allegra CJ, Kaye FJ, *et al.* Disruption of Myc-tubulin interaction by hyperphosphorylation of c-Myc during mitosis or by constitutive hyperphosphorylation of mutant c-Myc in Burkitt's lymphoma. *Mol Cell Biol.* 2000; **20**: 5276–5284.

36 Miyashita T, Harigai M, Hanada M, Reed JC. Identification of a p53-dependent negative response element in the *bcl-2* gene. *Cancer Res* 1994; **54**:3131–3135.

37 Reisman D, Elkind NB, Roy B, Beamon J, Rotter V. c-Myc trans-activates the p53 promoter through a required downstream CACGTG motif. *Cell Growth Differ* 1993; **4**:57–65.

38 Eischen CM, Packham G, Nip J, Fee BE, Hiebert SW, Zambetti GP, *et al.* Bcl-2 is an apoptotic target suppressed by both c-Myc and E2F-1. *Oncogene* 2001; **20**:6983–6993.

39 Beham A, Marin MC, Fernandez A, Herrmann J, Brisbay S, Tari AM, *et al.* Bcl-2 inhibits p53 nuclear import following DNA damage. *Oncogene* 1997; **15**:2767–2772.