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Suppression of c-*myc* expression and c-Myc function in response to sustained DNA damage in MCF-7 breast tumor cells

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Abstract

The topoisomerase II inhibitors teniposide (VM-26), doxorubicin, and amsacrine (*m*-AMSA), as well as ionizing radiation, induce a transient suppression of c-*myc* mRNA, which correlates with growth inhibition of MCF-7 breast tumor cells. To further assess the involvement of c-*myc* in the DNA damage-induced signal transduction pathways of the breast tumor cell, we determined the influence of sustained DNA damage on c-*myc* expression, c-Myc protein levels and c-Myc function. Continuous exposure of MCF-7 breast tumor cells to VM-26 induced DNA strand breaks that were sustained for at least 9 hr. DNA strand breakage was accompanied by a decline in c-*myc* transcripts and c-Myc protein levels by >90% after VM-26 exposure for 24 hr. The activity of a transcriptional target of the c-Myc protein, ornithine decarboxylase, was reduced by approximately 75% within 9 hr of DNA damage, in parallel to the declines in c-*myc* mRNA and protein levels. Extended exposure to VM-26 resulted in an initial loss of approximately 35% of the cell population followed by the death of additional cells such that by 72 hr only 50% of the cells were viable. Although apoptosis was evident 72 hr after initiating drug exposure [based on cell cycle analysis, terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) assays, and an assessment of cell morphology], the primary phase of cell killing, which occurred during the first 24 hr was non-apoptotic. These studies indicate that non-apoptotic pathways can also mediate cell death in the breast tumor cell and support the role of c-*myc* expression, c-Myc protein, and c-Myc function as elements of the DNA damage response pathway in the breast tumor cell.

Keywords: MCF-7; VM-26 (teniposide); DNA damage; c-Myc; Ornithine decarboxylase

1. Introduction

The c-*myc* oncogene is frequently overexpressed in human tumors. Regulation of c-*myc* expression and activity is associated with multiple cellular functions including the transition between G_0 and G_1 [1,2], entry and exit from S phase [3], progression of the cells along differentiation pathways [4,5], and the induction of apoptotic cell death [reviewed in Ref. 6]. Despite these pleiotropic effects, only a handful of transcription targets that are activated as a result of c-*myc* overexpression and which act as mediators of c-Myc function have been identified. These include the tyrosine phosphatase cdc25A [7], which targets cdk4/cyclin D complexes [8,9], cad [10], and ODC [11], the first and the rate-limiting enzyme of polyamine biosynthesis. All of these are direct c-Myc transcription targets that require consensus c-Myc:Max binding elements for their induction by c-Myc and by growth factors [7,11,12]. In addition, both ODC and cdc25A are mediators of c-Myc function, as ablation of cdc25A or ODC activity impairs c-Myc-induced apoptosis and blocks cell cycle progression in G_1 [7,13–15]. Interestingly, enforced expression of either target is also sufficient to augment the apoptotic program; yet, ODC is

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Abbreviations: ODC, ornithine decarboxylase; VM-26 (teniposide), 4′-demethylepipodophyllotoxin-4-(4,6-*O*-thenylidene-β-d-glucopyranoside); pRb, retinoblastoma protein; *m*-AMSA, amsacrine; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; and FBS, fetal bovine serum.

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not, like c-Myc, sufficient to promote continuous mitogen-independent cell-cycle progression [15]. Therefore, perturbation of the cell cycle is not strictly linked to the apoptotic program. In agreement with this concept, c-Myc-induced pathways that regulate apoptosis and cell cycle progression can be separated pharmacologically by treating myeloid cells with cyclic AMP analogues [15].

Transient exposure of MCF-7 breast tumor cells to the topoisomerase II inhibitors VM-26, doxorubicin, and *m*-AMSA, or to ionizing radiation, produces an early concentration-dependent (and in the case of radiation, a dose-dependent) reduction in c-*myc* transcript levels [16–19]. Suppression of c-*myc* mRNA by VM-26 (as well as by ionizing radiation) is a transient effect [18,19], which may be related to the transient nature of DNA strand breaks induced by acute exposure to topoisomerase II inhibitors or ionizing radiation [20–23]. VM-26, doxorubicin,[1] and ionizing radiation also promote pRb dephosphorylation in MCF-7 cells [24]. As hypophosphorylated pRb:E2F complexes repress c-*myc* transcription [25–28], this again suggests that regulation of c-*myc* could be linked to a DNA damage response pathway that arrests breast tumor cell growth.

It has been demonstrated that MCF-7 breast tumor cells are relatively refractory to apoptosis in response to DNA damaging modalities such as adriamycin or irradiation [17, 19,29–31]. This may be due, in part, to inactivation of caspase-3 in these cells [32], but other studies indicate that this resistance is common to a number of breast tumor cell lines [33,34]. To explore the linkages between the induction of DNA damage, the levels and activity of c-Myc protein, cell cycle arrest, and cell death, we continuously exposed MCF-7 cells to VM-26, thereby inducing sustained DNA strand breaks. Unlike the transient effects on c-*myc* expression observed after acute drug exposure or irradiation [18, 19], c-*myc* message levels declined and remained suppressed. Moreover, c-Myc protein levels were also suppressed, as was c-Myc protein activity, as measured by analysis of one of its targets, ODC, that is activated by c-*myc* overexpression [11,12]. Overall these studies demonstrate that c-*myc* is a critical component of signal transduction pathways responding to DNA damage.

2. Materials and methods

2.1. Cell culture and drug treatment

MCF-7 breast tumor cells were obtained from the NCI-Frederick Cancer Research Facility. SaOs-2 cells were obtained from Dr. Gerard Zambetti at St. Jude Children's Research Hospital. Cells were grown in Dulbecco's Minimum Essential Medium containing 10% FBS, at 37° in a humidified, 5% CO_2 atmosphere. Cells were plated in culture flasks at a density of $2 \times 10^4/cm^2$. The following day, the culture medium was replaced with medium containing 10 μM VM-26 (teniposide; provided by the Bristol-Myers Squibb Co.). This medium also contained DMSO, which was used as the drug solvent, at a final concentration of 0.2%; therefore, control cells were treated with medium containing 0.2% DMSO.

2.2. Alkaline unwinding (detection of single-strand breaks)

Bulk (single-strand) damage to DNA was determined using alkaline unwinding [35] as described previously [36]. This assay is based upon the differential binding and fluorescence of *bis*benzimide trihydrochloride (Hoechst 33258) to single-stranded and double-stranded DNA. F values, which represent the percent of double-stranded DNA remaining after a fixed period of alkaline denaturation, were converted to rad equivalence based on the standardization of DNA damage, using a cesium-137 irradiator that produced dose-dependent quantities of strand damage.

2.3. Northern blot analyses

The plasmid pMC413RC, containing a 1.4-kb human c-*myc* cDNA sequence [37], was provided by Dr. Eric Westin (Medical College of Virginia). The plasmid pHc-GAP, containing a 780-bp human GAPDH cDNA sequence, was obtained from the American Type Culture Collection. A plasmid containing a 1.6-kb murine ODC sequence was provided by Dr. Daniel Nathans (Johns Hopkins University). Total cellular RNA was isolated using the RNA STAT-60 procedure as described by the manufacturer (Tel-Test "B," Inc.). Gel electrophoresis, probe radiolabeling, and hybridization were performed as described previously [18].

2.4. Western blot analyses

Total protein from cell lysates was separated by SDS–PAGE and transferred to nitrocellulose membranes (Nitro-Bind; Micron Separations, Inc.). c-Myc protein was detected using culture medium from 9E10 hybridoma cells, which produce a human c-Myc-specific monoclonal antibody [38]. Bound antibody was detected with a horseradish peroxidase-labeled goat anti-mouse IgG antibody (Kirkegaard & Perry) and chemiluminescence (SuperSignal Substrate; Pierce).

2.5. ODC activity

Cells (1×10^7) were collected by trypsinization and washed twice in ice-cold PBS [137 mM NaCl, 2.7 mM KCl, 1 mM Na_2HPO_4, 1.8 mM KH_2PO_4, (pH 7.2)], and then pellets were quick-frozen in liquid nitrogen. Pellets were then suspended in 500 μL of ODC assay buffer [10 mM

[1] Di Y-M, Holt S, Gewirtz DA, Manuscript in preparation.

Tris–HCl (pH 7.4), 0.1 mM EDTA, 2.5 mM dithiothreitol, 0.2 mM pyridoxal-5-phosphate], and lysed by three cycles of freeze–thawing; debris was removed by centrifugation. Then ODC assays were performed in duplicate as described previously [14].

2.6. Determination of cell viability

The number of viable cells was determined by trypan blue dye exclusion as described previously [17,19].

2.7. Clonogenic analysis

Cells treated with VM-26 were trypsinized under sterile conditions, and plated in triplicate in six well culture dishes at 1000 cells/plate. After 10 days, cells were fixed with methanol, air-dried for 1–2 days, and stained with 0.1% crystal violet. For determining cell survival, groups of 50 or more cells were counted as colonies and were normalized to the initial cell number plated.

2.8. Cell cycle analysis

Cells were collected and resuspended in PIF solution (3.8 mM sodium citrate, 0.05 mg/mL of propidium iodide, 0.1% Triton X-100, 9 Kunitz Units/mL of RNase A) and incubated for 45 min at 4°. DNA content per cell was determined by cytofluorimetry using a Becton-Dickinson FACScan model FC.

2.9. Light microscopy analysis of cell morphology

Both adherent and nonadherent cells were collected, deposited onto cytocentrifuge slides, stained using a Wright stain technique (Diff-Quik Stain Set; Dade International, Inc.), and analyzed by light microscopy for the presence of cyto-architectual features of apoptosis.

2.10. TUNEL assay

Both adherent and nonadherent cells were collected onto cytocentrifuge slides and fixed with 4% formaldehyde in PBS followed by acetic acid:ethanol (1:2). After washing with PBS, cells were incubated with 1 mg/mL of bovine serum albumin in PBS for 30 min at room temperature, then washed again with PBS. DNA fragment ends were labeled by incubating the cells with 500 μM fluorescein-12-dUTP and 0.25 U/μL of terminal deoxynucleotidyl transferase (Boehringer Mannheim) in 1X TdT Reaction Buffer [200 mM potassium cacodylate, 25 mM Tris–HCl, 0.25 mg/mL of bovine serum albumin, 2.5 mM cobalt chloride] for 1 hr at 37°. Cells were washed with PBS and mounted in Vectashield (Vector Laboratories, Inc.).

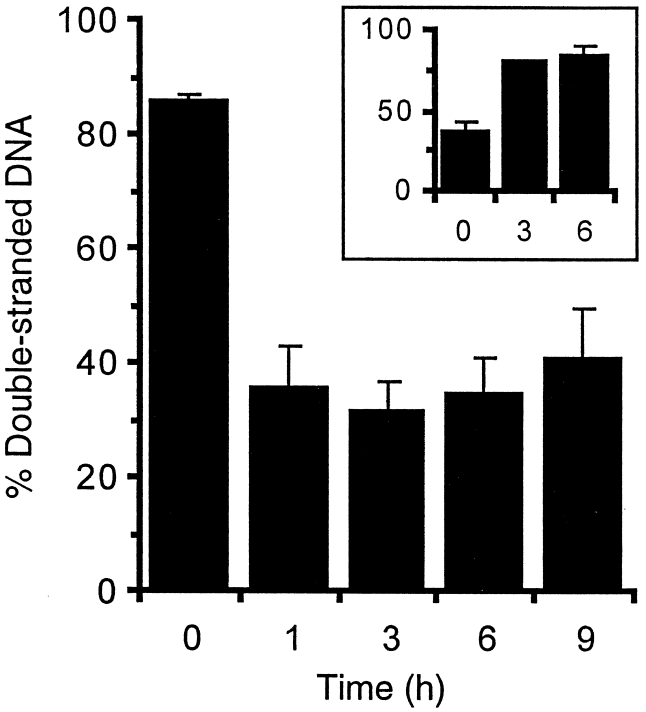

Fig. 1. Induction of DNA strand breaks in MCF-7 cells by VM-26. Cells were incubated in the continuous presence of 10 μM VM-26. DNA strand breaks were assessed at the intervals indicated using the technique of alkaline unwinding. Data shown represent the means ± SEM for three independent experiments. Inset: The induction and reversal of DNA strand breaks after acute exposure to VM-26. MCF-7 cells were exposed to 10 μM VM-26 for 3 hr. Drug was removed, cells were washed free of residual drug, and DNA strand breaks were assessed at the intervals indicated after incubation of the cells in drug-free medium. Data shown represent the means ± range from two independent experiments.

3. Results

3.1. Suppression of *c*-myc *expression associated with persistent DNA strand breaks*

Acute exposure of MCF-7 breast tumor cells to the topoisomerase II inhibitors VM-26, *m*-AMSA, and doxorubicin, or to ionizing radiation, results in a time- and dose-dependent suppression of c-*myc* mRNA levels [16–19]. By contrast, exposure of MCF-7 cells to vincristine, a microtubule inhibitor that does not produce DNA damage, failed to down-regulate c-*myc* mRNA levels (unpublished data). The suppression of c-*myc* expression by VM-26 is a transient effect [18], and recovery of c-*myc* expression also occurred in MCF-7 cells after exposure to ionizing radiation (unpublished data). As the transient suppression of c-*myc* mRNA appeared to be related to the induction and reversal of DNA strand breaks, we examined the effects of sustained DNA damage on c-*myc* expression following chronic exposure of MCF-7 cells to VM-26. DNA damage induced in MCF-7 cells by continuous exposure to VM-26 was sustained throughout the course of the experiment (9 hr; Fig. 1). In contrast, acute exposure (3 hr) to VM-26 produced

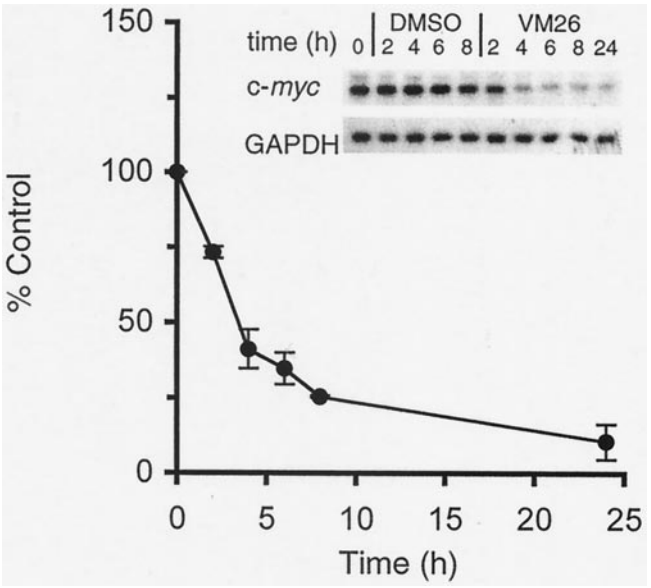

Fig. 2. Suppression of c-*myc* expression by VM-26 in MCF-7 cells. Cells were treated with 10 μM VM-26 for the intervals indicated. Total cellular RNA was isolated, and c-*myc* and GAPDH mRNA levels were evaluated by northern blotting. Data shown represent the means ± SEM from three independent experiments. All data for c-*myc* expression were normalized for GAPDH expression and are presented as a percentage of corresponding DMSO-treated controls. Inset: representative northern blots.

DNA strand breaks that were almost fully reversed within 3 hr after removal of the drug (Fig. 1, inset).

The capacity of chronic exposure to VM-26 to suppress the expression of c-*myc* was assessed over a time frame of 24 hr. Within 4 hr, c-*myc* mRNA levels were reduced to approximately 40% of their initial value. By 24 hr, expression was reduced to 10% of the initial value (Fig. 2). This sustained repression of c-*myc* mRNA is thus distinct from the rapid reversal of the c-*myc* suppression observed after acute exposure to VM-26 [18], and is consistent with the concept that the effects of VM-26 on c-*myc* expression reflect the sustained induction of DNA damage.

*3.2. Reduction in c-*Myc *protein levels by chronic exposure to VM-26*

The pronounced suppression of c-*myc* mRNA levels by VM-26 was reflected by a corresponding reduction in levels of the c-Myc oncoprotein (Fig. 3A). c-Myc protein levels were profoundly reduced by continuous exposure to VM-26; after 4 and 8 hr, c-Myc protein levels were approximately 30% of their initial value and by 24 hr, c-Myc protein levels had been reduced by greater than 90%. This suppression of c-Myc protein levels was sustained for at least an additional 24 hr (Fig. 3A). To rule out nonspecific effects of VM-26 on the suppression of protein synthesis and/or the blockade of protein degradation, we assessed the influence of VM-26 on the levels of E2F4, a protein associated with cell-cycle regulation [39]. The levels of E2F4 were not reduced by VM-26 treatment (Fig. 3B).

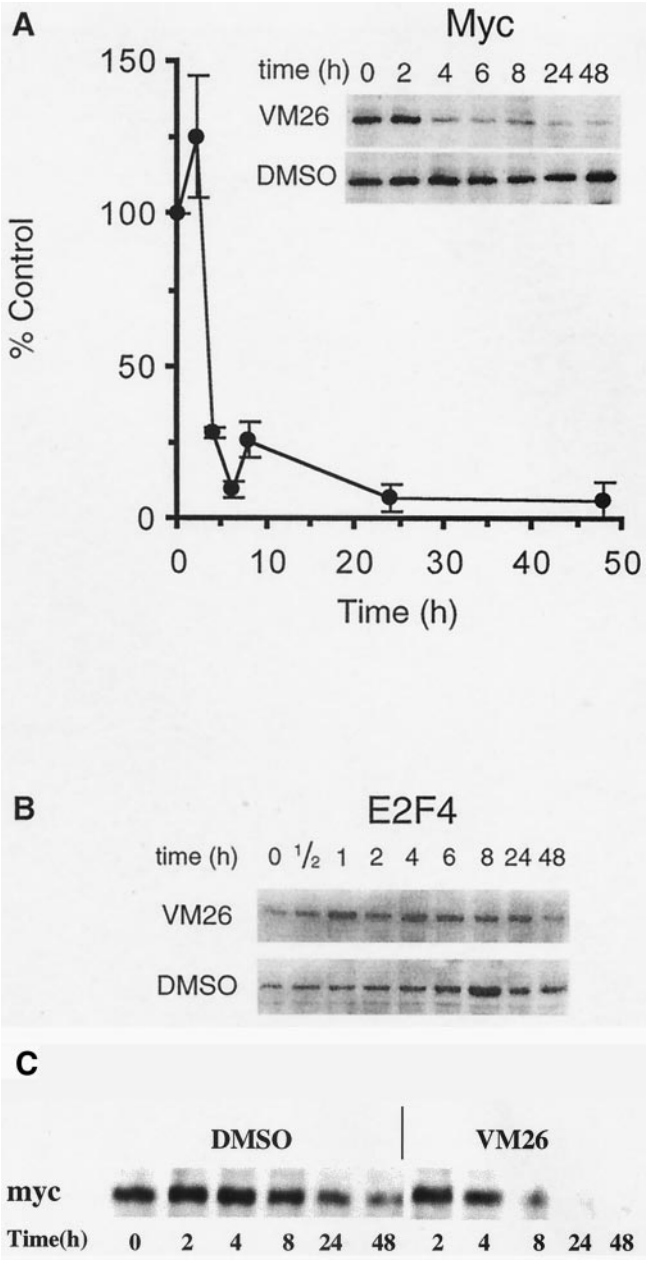

Fig. 3. Suppression of c-Myc protein levels by VM-26 in MCF-7 and SaOs-2 cells. Cells were exposed continuously to 10 μM VM-26. Total cellular protein was isolated, and (A) c-Myc or (B) E2F4 protein levels were determined in MCF-7 cells by western blotting. Data in (A) are presented as percent of c-Myc protein at time 0 and represent the means ± SEM from three independent experiments. Inset: representative western blots. (B) Representative western blots showing E2F4 protein. (C) Effect of VM-26 on c-Myc protein levels in SaOs-2 cells.

We have reported previously that in MCF-7 cells, VM-26 (through induction of p53) activates transcription of the general cyclin-dependent kinase inhibitory protein $p21^{waf1/cip1}$, which, in turn, leads to the accumulation of hypophosphorylated retinoblastoma protein (Rb) [24]. Both p53 [40,41] and pRb [25–28] have been shown to repress c-*myc* transcription. To determine whether p53 and/or Rb function were required for the suppression of c-*myc* expres-

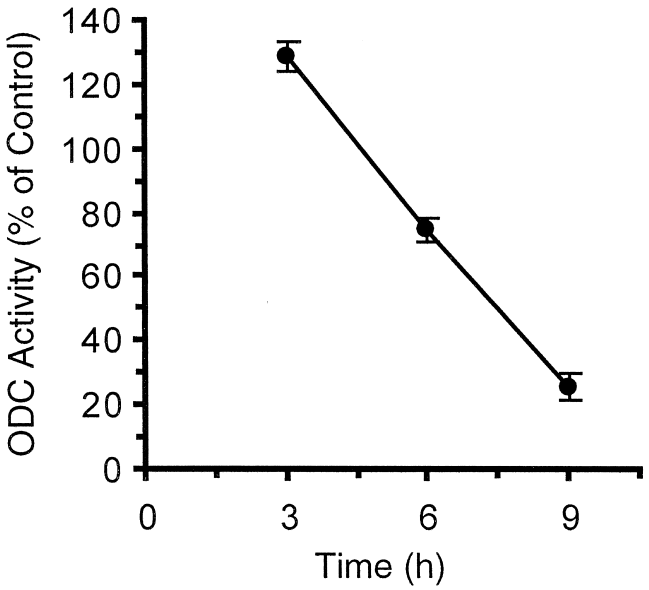

Fig. 4. Suppression of ODC activity by VM-26 in MCF-7 cells. MCF-7 cells were exposed continuously to 10 μM VM-26; cells were isolated at the intervals indicated, and ODC activity was determined. Each value was normalized for protein content and represents the means ± SEM from four independent experiments. The initial baseline ODC value was 2274 ± 457 pmol CO_2/hr/mg protein.

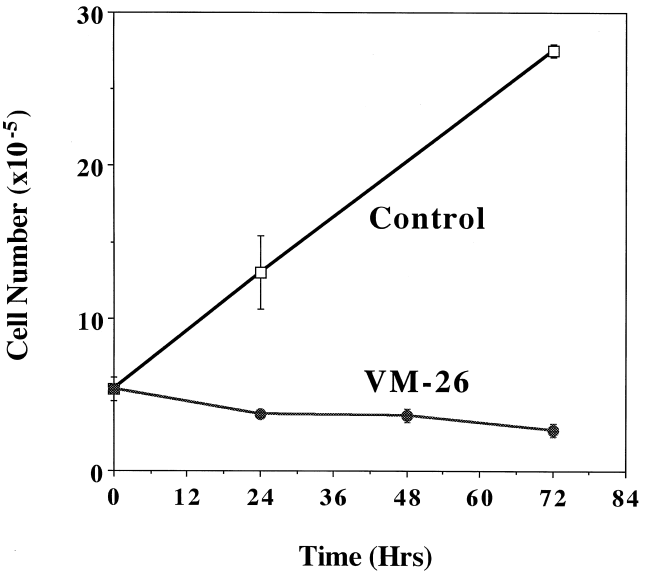

Fig. 5. Influence of VM-26 on the number of viable MCF-7 cells. MCF-7 cells were exposed continuously to 10 μM VM-26. At the intervals indicated, the number of viable cells was determined by exclusion of Trypan Blue dye. Data are shown as a percent of the starting cell number and represent the means ± SEM from three independent experiments.

sion by VM-26, similar studies were also performed in the p53-mutated, Rb-null SaOs-2 cell line. As shown in Fig. 3C, continuous exposure to VM-26 reduced c-Myc protein levels in the SaOs-2 cells; however, the decline was somewhat slower than in MCF-7 cells in that c-Myc protein levels were reduced by ~ 20% after 4 hr (compared with 70% in MCF-7 cells), 50% after 8 hr (compared with 75% in MCF-7 cells), and 70% after 24–48 hr (compared with > 95% in MCF-7 cells). In contrast, levels of β-actin were not affected by VM-26 (not shown).

3.3. Inhibition of ODC, the c-Myc-specific transcription target, by VM-26

To determine whether the profound suppression of c-Myc protein levels by VM-26 in MCF-7 cells was also reflected by a corresponding reduction in c-Myc function, we assessed the effects of VM-26 on the activity of ODC, a well-characterized transcription target induced by c-*myc* overexpression [11,12]. VM-26 produced a pronounced, time-dependent reduction in ODC activity in MCF-7 cells, such that by 9 hr, activity was reduced to 25% of the corresponding control levels (Fig. 4); this reduction corresponded closely with the reduction in c-*myc* message and protein levels observed at this interval. Reductions in ODC activity followed parallel reductions in ODC transcripts (data not shown). Therefore, the induction of DNA damage by VM-26 results in the suppression of c-*myc* mRNA and protein levels and concomitantly decreases the expression of the c-Myc target, ODC.

3.4. Cell death and promotion of apoptosis by VM-26

Although MCF-7 breast tumor cells are relatively refractory to the induction of apoptosis, at least by agents that induce damage to DNA [17,19,29–31,33,42–44], it was possible that the continuous exposure to VM-26 and the sustained induction of DNA strand breaks might be sufficient to produce cell killing. The primary decline in cell number (of ~35%) was evident within the first 24 hr followed by a more gradual decline over the succeeding 48 hr, such that by 72 hr cell number was reduced to approximately 50% of the initial value (Fig. 5). Continuous exposure to VM-26 also resulted in a 76% reduction in clonogenic survival (data not shown).

Cell cycle analysis of MCF-7 cells exposed to VM-26 demonstrated a time-dependent increase in the S and G_2/M phase populations and a corresponding decline in the proportion of cells in G_0/G_1 (Fig. 6). The accumulation of cells in S phase was substantiated by studies performed in the presence of nocodazole, to prevent leakage from G_2/M (data not shown). Starting at 24 hr, there was evidence of a small sub-$G_{0/1}$ population in drug-treated cells, which increased slightly at 48 hr and quite dramatically at 72 hr, consistent with the promotion of apoptosis. Cells treated with DMSO demonstrated no evidence of a sub-$G_{0/1}$ population (data not shown).

The capacity of VM-26 to induce apoptosis in MCF-7 cells was substantiated by an analysis of cell morphology and by the use of the TUNEL end-labeling assay. TUNEL analysis indicated that there was a significant increase in fluorescent (apoptotic) cells at 72 hr of exposure to VM-26 (Fig. 7). Morphological examination also demonstrated un-

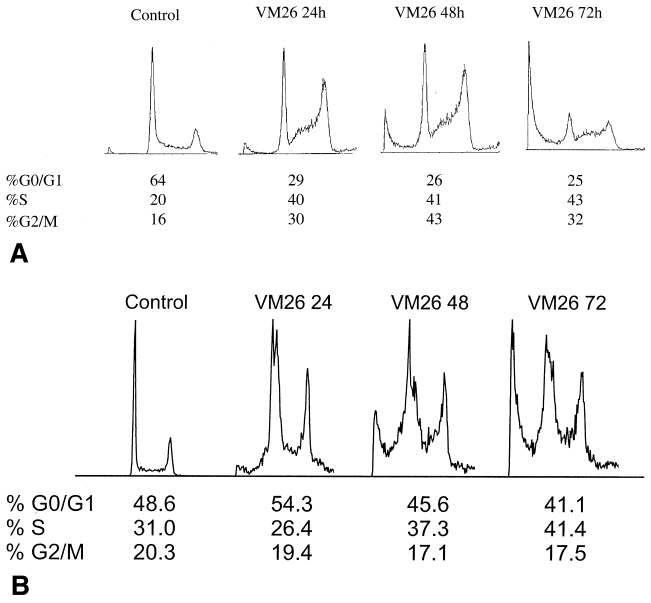

Fig. 6. Influence of VM-26 on cell cycle distribution. MCF-7 (A) or SaOs-2 (B) cells were exposed continuously to 10 μM VM-26, and cell cycle distribution was determined at the intervals indicated. Numbers indicate the percentage of cells in G_0/G_1, S, and G_2/M phases.

equivocal evidence for apoptotic cell death, again primarily after 72 hr of exposure to VM-26 (Fig. 8).

In SaOs-2 cells, continuous exposure to VM-26 led to a decline in the G_0/G_1 population coupled with an increase in cells primarily in the S phase fraction (Fig. 6B). In addition, a significant fraction of sub-$G_{0/1}$ cells was evident within 24 hr of drug exposure and continued to increase thereafter (Fig. 6B). Concomitantly, we observed a decline in the number of viable cells, with a 40% reduction after 48 hr and an 80% decline by 72 hr (data not shown).

4. Discussion

4.1. c-Myc and ODC as targets suppressed by the DNA damage response pathway

We utilized the strategy of inducing sustained (topoisomerase II mediated) DNA strand breaks to investigate the influence of DNA damage on c-*myc* expression, c-Myc function, growth arrest, and cell death in MCF-7 breast tumor cells. A continuous decline in c-*myc* expression accompanied the induction of sustained DNA strand breaks; this finding is consistent with the hypothesis that the suppression of c-*myc* expression is related to the induction of DNA damage. In particular, c-*myc* expression is at least partially restored in association with the reversal of strand breaks in cells acutely exposed to VM-26 [18]; in contrast, sustained suppression of c-*myc* is observed with continuous exposure to VM-26 where DNA damage is sustained. These sustained strand breaks are presumably being reversed and reformed as a consequence of the induction of DNA–topoisomerase II cleavable complexes, as well as secondary breaks that result from either the processing of the initial lesions or the collision of the replication fork with trapped cleavable complexes [45].

As would be expected, the suppression of c-*myc* expression was accompanied by a corresponding reduction in c-Myc protein levels. The profound reduction in ODC expression and activity is consistent with the concept that c-Myc protein function is also compromised. These findings therefore support the concept of c-*myc* as a target of DNA damage signal transduction pathways. This concept is further supported by a similar suppression of c-Myc-levels by VM-26 in the SaOs-2 cells.

It is important to emphasize that c-Myc is not the sole factor that regulates ODC activity. Utilizing inducible expression of a dominant negative form of c-*myc*, Packham and Cleveland [12] identified both c-Myc-dependent and c-Myc-independent pathways for modulation of ODC. Bush *et al.* [46] reported that ODC (as well as other putative c-Myc targets) was not misregulated in c-*myc* null cells, which indicates that maintenance of ODC function is not dependent upon c-Myc. Therefore, the concurrent suppression of c-*myc* expression, c-Myc protein levels and ODC activity by VM-26 in this report does not provide proof that the influence of VM-26 on ODC is directly mediated through c-Myc.

The suppression of c-*myc* expression by VM-26 could be a consequence of: (a) preferential induction of damage within regions of the c-*myc* gene [36]; (b) the increase in p53 protein levels, as p53 can suppress c-*myc* transcription [40,41]; and/or (c) repression of c-*myc* transcription by the Rb:E2F complex [25–28], which forms as a consequence of the increase in levels of p53 and its target $p21^{waf1/cip1}$, which leads to pRb dephosphorylation. The observation that levels of the c-Myc protein were also reduced by continuous exposure to VM-26 in the p53 mutant, Rb null SaOs-2 cell line rules out a role for either p53 or Rb–E2F complex formation at least in these cells. Thus, VM-26 must suppress c-*myc* expression through other signaling pathways in SaOs-2 cells.

4.2. Apoptotic response to DNA damage in MCF-7 breast tumor cells

We and others have reported that MCF-7 cells are relatively refractive to apoptosis in response to DNA damage [17,19,29–31,42–44]. Here, VM-26 produced a slow but sustained cell killing; yet, even with continuous drug exposure, more than 50% of the cell population remained viable after 72 hr. Furthermore, there was minimal evidence for apoptosis during the first 24 hr after exposure of the cells to VM-26, when the greatest reduction in cell number was evident. In contrast, greater than 80% of the SaOs-2 cell population was eliminated by a similar drug exposure and died through apoptosis in response to VM-26. Consequently, as described previously in the case of adriamycin [17,42], the initial response of MCF-7 cells even to prolonged exposure to VM-26 is non-apoptotic cell death; this

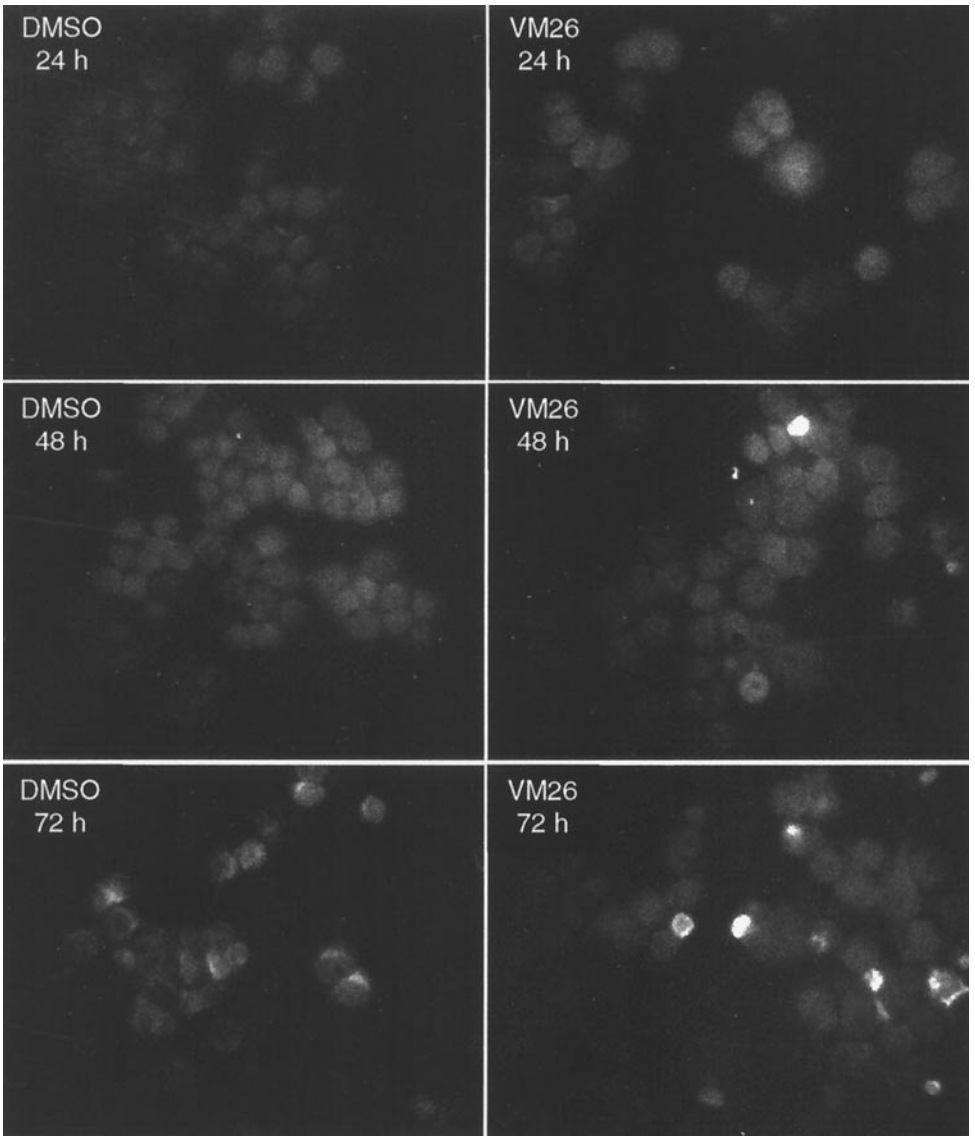

Fig. 7. *In situ* analysis of DNA fragmentation by fluorescent DNA end-labeling. Cells were exposed to 10 μM VM-26 or solvent alone (DMSO). After the indicated intervals, cells were collected on glass microscope slides and labeled according to the TUNEL assay. Fluorescent cells contained fragmented DNA.

is succeeded by a prolonged growth arrest accompanied by a small degree of apoptotic cell death.

The impaired apoptotic response of MCF-7 cells may be due, at least in part, to the fact that MCF-7 cells fail to express a functional form of caspase 3 [32,47]. Nevertheless, others have demonstrated unequivocal apoptotic responses to a variety of non-DNA damaging agents in these breast tumor cells [48–51]. Sokolova *et al.* [52] described substantive DNA fragmentation in MCF-7 cells after continuous prolonged exposure of MCF-7 cells to VP-16 (an analog of VM-26)—similar to our findings that apoptosis is evident after continuous exposure to VM-26.

Overexpression of either c-Myc or ODC is associated with the induction of apoptotic cell death [15,53–56]. The pronounced suppression of c-*myc* expression and c-Myc protein function would be expected to block any c-Myc-dependent apoptosis. However, the induction of apoptosis is a complex process, and it is likely that other factors, such as the high levels of Bcl-2 and Bcl-x_L reported in MCF-7 cells [57,58] or the activation of anti-apoptotic pathways such as NF-κB [59,60], may influence susceptibility of MCF-7 cells to apoptotic cell death in response to DNA damage.

4.3. Defective regulation of the G_1 *checkpoint in response to DNA damage in breast tumor cells*

An additional observation worthy of note is that MCF-7 cells (like SaOs-2 cells) also fail to undergo G_1 arrest, despite the fact that VM-26 also up-regulates levels of p53 and $p21^{waf1/cip1}$, and pRb is dephosphorylated in the breast tumor cell line [24]. Here, we have demonstrated that VM-26 also suppresses c-*myc* expression, c-Myc protein levels and c-Myc function, yet cells still fail to arrest in G_1, as normal cells would in the absence of c-Myc [53]. We

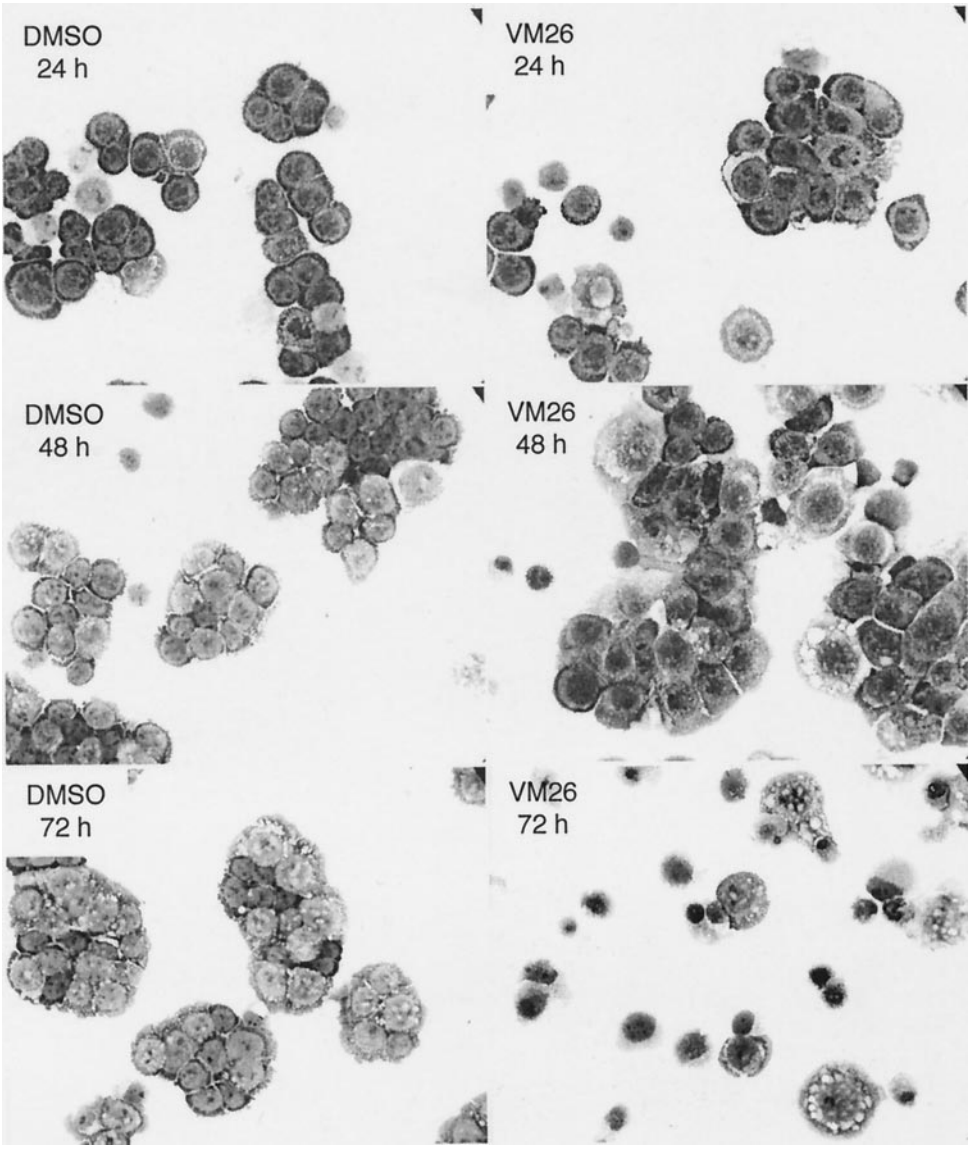

Fig. 8. Morphological analysis of MCF-7 cells after continuous exposure to VM-26. Cells were exposed to 10 μM VM-26 or solvent alone (DMSO) for the intervals indicated, and then were collected, and counterstained as described in "Materials and methods." The small, densely stained cells are indicative of apoptotic cells.

have hypothesized that the lack of G_1 arrest may be related to the failure of VM-26 to suppress E2F activity [24]. Shapiro *et al.* [61] have demonstrated that the capacity of Calu-1 non-small cell lung cancer cells to undergo G_1 arrest can be restored by the induction of $p16^{INK4A}$ expression. Since many breast tumor cells, including MCF-7 cells, are defective in p16 expression or function [62], a permissive role for p16 may be required for promotion of G_1 arrest through the suppression of E2F activity in p53 wild-type cells.

The accumulation of MCF-7 cells exposed to VM-26 in G_2 (and the absence of G_2 arrest in the SaOs-2 cells) suggests that p53 status may influence this response. That is, the arrest in G_2 may be related to the reported capacity of p53 to maintain growth arrest in G_2 [63,64]. The absence of G_1 arrest in MCF-7 cells, despite the profound reduction in both c-Myc protein and ODC activity and the dephosphorylation of Rb, attests to defects in checkpoint function at a point downstream of p53. The delayed and limited promotion of apoptosis is indicative of an additional defect in cellular signaling (e.g. caspase-3 inactivation) that may be exacerbated by reduced c-Myc protein function. It is possible that complementary defects in the regulation of the G_1 checkpoint and apoptosis may provide a mechanism for breast tumor cells to evade both chemotherapy and radiotherapy.

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