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ORIGINAL ARTICLE

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Bisimidazoacridones induce a potent cytostatic effect in colon tumor cells that sensitizes them to killing by UCN-01

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Abstract *Purpose*: To determine the ability of WMC26, a prototypic bisimidazoacridone (BIA), to induce apoptosis in sensitive colon adenocarcinoma cells and to advance the hypothesis that cancer cells that are growth-arrested by WMC26 are predisposed to undergo apoptotic death by abrogators of cell cycle checkpoints. *Methods*: The antiproliferative activity of WMC26 was examined in detail by a 4-day MTT assay, cell counting, BrdU incorporation and a two-color LIVE/DEAD assay. To detect apoptosis a number of established techniques were used, including gel electrophoresis, flow cytometry, and confocal laser microscopy of treated cells. The activity of senescence-associated β-galactosidase in treated cells was also analyzed. *Results*: WMC26, at physiological concentrations, induced complete and longlasting growth arrest of HCT116 cells in culture but did not trigger cell death. The growth-arrested cells (blocked at G_1 and G_2/M cell cycle checkpoints) did not synthesize DNA but were metabolically active and had intact plasma membranes. Although they resembled the senescence-like phenotype reported to be induced by treatment with some antitumor agents, the cells did not express senescence-associated β-galactosidase, an indicator of the senescence-like state. Treatment of WMC26 growth-arrested cells with 1 μM UCN-01, an abrogator of the G_2/M checkpoint, caused a very rapid (1 h) change in morphology and cell death within 72 h. *Conclusions*: BIAs do not induce apoptosis in sensitive colon tumor cells. They are highly cytostatic but only marginally toxic to the cells even at concentrations 100-fold higher than those sufficient for complete growth arrest. In this respect WMC26 differs from some other DNA-interacting antitumor agents that produce cell growth arrest at low concentrations but are toxic at higher doses. The complete growth arrest induced by WMC26 in colon cancer cells sensitized them to apoptotic death induced by UCN-01. This finding suggests that a combination of WMC26 and cyclin-dependent kinase inhibitors may be an attractive treatment method for colon cancer that utilizes the highly tumor-selective activity of WMC26.

Key words Bisimidazoacridones · Colon cancer · Cell cycle · Cytostasis

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Introduction

Bisimidazoacridones (BIAs) and related compounds were designed in our laboratory as potential bisintercalating anticancer drugs [4]. The general structure of a BIA consists of two planar intercalating moieties connected via an aminoaliphatic linker, which may vary in length and rigidity. Some of the compounds show potent but selective activity against colon cancer and leukemias, while others, which differ slightly in structure or linker length, are totally inactive or nonselective in the NCI screen against 60 human tumor cell lines. WMC26, a prototypic BIA (Fig. 1), arrests the growth of HCT116 xenografts in nude mice [4]. Although physicochemical studies have shown that BIAs do not bind to DNA by bisintercalation, the presence of two planar aromatic ring systems has been found to be crucial for selectivity and activity. Our previous studies suggested that components of the nucleotide excision machinery may be a target for some BIAs [9] and/or that the compounds directly interfere with transcriptional machinery [26].

It has been recognized recently that the most of the effective antitumor agents kill cancer cells by inducing apoptosis in spite of differing molecular targets [7, 12, 18]. As a part of our studies on the mechanism of action of BIAs, we wished to determine whether WMC26 triggers apoptotic processes in sensitive tumor cells. The HCT116 colon adenocarcinoma cell line was selected for

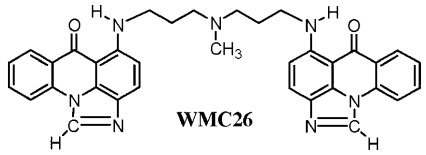

Fig. 1 Chemical structure of WMC26, a prototypic bisimidazoacridone

this work as representative of human colon tumors, which had previously been shown to be sensitive to BIA treatment [4, 9].

Materials and methods

Chemicals and cells

All chemicals and cell culture reagents were from Sigma (St. Louis, Mo.). Tissue culture materials were from Corning (Acton, Mass.). The synthesis of WMC26 has been described previously [4]. UCN-01 was kindly provided by the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, NCI. For both drugs, stock solutions (5 m*M*) in DMSO were prepared freshly for each experiment. Stock of UCN-01 was directly diluted with medium, and stock of WMC26 was initially diluted ten times with distilled water and then with the culture medium to the desired concentration. HCT116 cells were obtained from the American Type Culture Collection and grown in DMEM containing 10% fetal bovine serum and standard antibiotics. The control cells were untreated.

Cell growth inhibition by WMC26 and cytotoxic effect in combination with UCN-01

The capacity of WMC26 to interfere with the growth of the HCT116 cells was determined using the MTT-based, CellTiter96 nonradioactive cell proliferation assay (Promega, Madison, Wis.), according to the manufacturer's protocol. An incubation time of 96 h and 2500 cells/well (96-well plates) were used in this assay. An assay was also performed on extra reference plates to determine the cell population density at time 0 (T_0). Similar conditions were used to determine the cytotoxic effect of UCN-01 in cells pretreated with WMC26. In this experiment, cells were initially exposed for 48 h to 100 n*M* WMC26. Next, the medium was changed to a fresh one containing various concentrations of UCN-01, the cells were incubated for 72 h, and the MTT assay was performed. As a negative control, HCT116 cells were incubated for 120 h with the same concentrations as above of UCN-01, without pretreatment with WMC26.

In addition, the effect of WMC26 on DNA synthesis was examined in terms of bromodeoxyuridine (BrdU) incorporation using a BrdU cell proliferation assay (Oncogene Research Products, Cambridge, Mass.). In this assay, 2500 cells/well were allowed to attach for 24 h, and were then treated with various concentrations of the drug for 24 h and incubated with BrdU for 24 h. The level of incorporated BrdU was measured immunochemically according to the manufacturer's protocol.

Cell number determination and cell cycle analysis

To determine the capacity of WMC26 to induce cell death, the number of viable cells was assessed after a short treatment with WMC26, followed by a prolonged incubation in fresh medium. A suspension of 0.5×10^6 cells in 8 ml medium was placed in 25-cm^2 T flasks and allowed to attach for 24 h. The cells were then exposed for 2 h to 1 μ*M* WMC26. After removal of the drug the cells were washed with PBS. Fresh medium (8 ml) was added and incubation at 37 °C in an atmosphere containing 5% CO_2 and in complete humidity was continued for an additional 6 days. At appropriate intervals, treated and control cells were released from flasks by incubation with trypsin (0.05 mg/ml)/EDTA (0.02 mg/ml) for 5 min at 37 °C, collected in ice-cold PBS, combined with the removed medium that possibly contained floating cells, and centrifuged at 4 °C. Cell pellets were resuspended in PBS containing 1% fetal bovine serum. A small aliquot of the suspension was mixed with trypan blue and cells were counted by a hemocytometer. The remaining cells were fixed and stained for fluorescence-activated cell sorting according to standard procedures [5]. Briefly, cells were fixed in 70% ethanol and washed twice with PBS containing 10% fetal bovine serum. Then the cells were treated with RNase (1 U/10^6 cells) for 30 min at 37 °C, chilled on ice, and stained overnight with propidium iodide (50 μg/10^6 cells) in the cold. Fluorescence histograms were obtained on a Coulter EPICS753 cell sorter using an argon laser, and the mean peaks were analyzed.

Viability/cytotoxicity assay

To additionally verify that WMC26 does not trigger cell death a LIVE/DEAD viability/cytotoxicity kit (Molecular Probes, Eugene, Ore.) was used according to the manufacturer's fluorescence microscopy protocol provided with the kit.

Caspase-3 fluorogenic assay

Caspase-3 activity was measured using a caspase-3 assay kit (Pharmingen, San Diego, Calif.) and an Eppendorf tube method. Briefly, cells were harvested and washed with PBS. 3×10^6 cells were pelleted and lysed with 300 μl of the cell lysis buffer provided with the kit. Cell lysate (100 μl) was added to reaction tubes, one containing 10 μl reconstituted Ac-DEVD-AMC (caspase-3 fluorogenic substrate) in 1 ml HEPES buffer, and a second containing the same amount of Ac-DEVD-AMC and 10 μl reconstituted Ac-DEVD-CHO (caspase-3 inhibitor) in 1 ml HEPES buffer, and the reaction mixtures were incubated for 1 h at 37 °C. The fluorescence of 7-amino-4-methylcoumarin (AMC) liberated from Ac-DEVD-AMC was measured using a spectrofluorometer (excitation 380 nm, emission 438 nm). The level of caspase-3 was determined by subtracting the fluorescence with the blocker from the reaction without blocker.

β-Galactosidase staining

Staining for senescence-associated β-galactosidase (SA-β-Gal) was performed as described previously [6]. Briefly, after 12 days of cell growth in medium containing 100 n*M* of WMC26, the culture medium was removed, and the cells were washed with PBS, and fixed for 5 min with 3% formaldehyde. After fixation, cells were washed with PBS and incubated for 16 h at 37 °C with fresh β-galactosidase stain solution at pH 4.0 or pH 6.0. The same procedure was applied to untreated cells. After incubation the cells were washed three times with PBS and examined under an inverted phase contrast microscope.

Results

Cell growth inhibition

The growth of HCT116 cells was inhibited in a dose- and time-dependent manner at concentrations of WMC26 below 100 n*M*, as measured by the cell proliferation MTT assay (Fig. 2). At these concentrations the inhi-

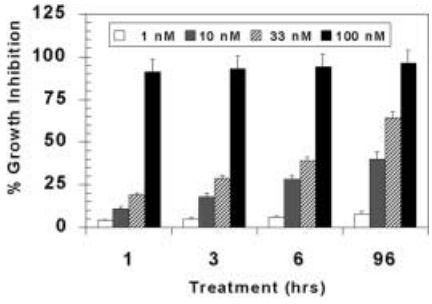

Fig. 2 Effect of low concentrations of WMC26 on the growth of HCT116 cells. Cells grown in 96-well plates were exposed to various concentrations of the drug for different times, and then incubated for 96 h. The effect of the treatment was determined using the MTT cell proliferation assay and was calculated as percent growth inhibition: $100 \times [1 - (T - T_0)/(C - T_0)]$, where T_0, T, and C are the absorbance values at 540 nm for time 0, and in treated and control cells, respectively. The data points are the means of three independent experiments

bitory effect was also subject to the number of cells used in the experiments. At 100 n*M* or higher concentrations of WMC26, exposures as short as 2 h were sufficient to produce total growth inhibition in the entire cell population after 24 h of incubation. The drug-induced cell growth arrest persisted for more than 2 weeks. However, it could be reversed even after several weeks in culture by frequent changes of the culture medium.

Cell counting experiments (Fig. 3A) showed that cells exposed for 2 h to 1 μ*M* WMC26 completely stopped dividing after about 24 h, and their number was constant for the next 6 days. The MTT assay performed at different time-points also suggested cell growth arrest (Fig. 3B), which was accompanied by a total shut-down of DNA synthesis, as evidenced by the BrdU incorporation experiment (Fig. 4). The MTT assays cannot distinguish between complete growth arrest and a situation where the cells die at the same rate as new cells are produced. However, the results of cell counting and BrdU incorporation taken together showed that WMC26 causes complete growth arrest without subsequent cell death.

Cell cycle analysis

FACS analysis of the cells used in the cell counting experiment showed arrest in G_1 and G_2/M phases and a dramatic reduction in the S-phase population during the 24 h after treatment (Fig. 5B). The G_1 to G_2/M peak ratio remained constant for the next 6 days. During that time WMC26 did not trigger apoptotic cell death as the sub-G_1 population was essentially constant and comparable to that in the untreated cells.

The LIVE/DEAD viability assay

This two-color assay clearly distinguishes between live cells with intracellular esterase activity (green fluorescence from calcein produced by enzymatic conversion of nonfluorescent calcein AM; Fig. 6A) and dead cells with damaged membranes (red fluorescence from ethidium dimer; Fig. 6B). The LIVE/DEAD test showed that even after continuous exposure to 1 μ*M* WMC26 for 12 days,

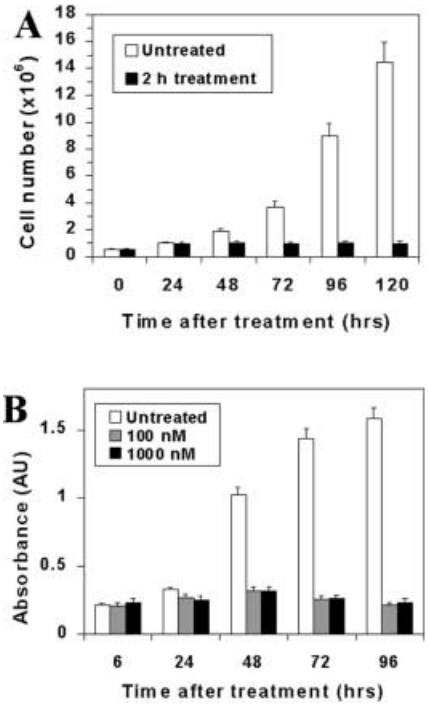

Fig. 3A, B Cell viability and cell growth inhibition after exposure to higher concentrations of WMC26. **A** Total cell growth inhibition determined by cell counting. Cells (5×10^5) were plated in 25-cm^2 flasks and, after attachment, exposed for 2 h to 1 μ*M* drug. Viable cell numbers were determined at different time-points by the trypan blue exclusion method and are averages of two separate experiments. Since the number of nonviable cells was negligible, the data reflect the total number of cells. **B** Total growth inhibition determined by the MTT cell proliferation assay. Cells grown in 96-well plates were exposed for 2 h to 0.1 μ*M* or 1 μ*M* WMC26. The viability of the cells was tested at different time-points by the MTT reaction. The results are averages of three independent experiments

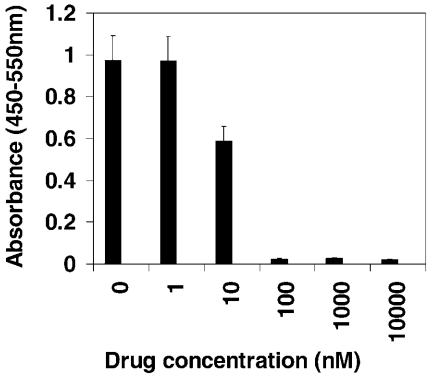

Fig. 4 Effect on DNA synthesis caused by exposure for 24 h to WMC26 evaluated in terms of the level of incorporation of BrdU, measured immunochemically. The experiment was performed twice

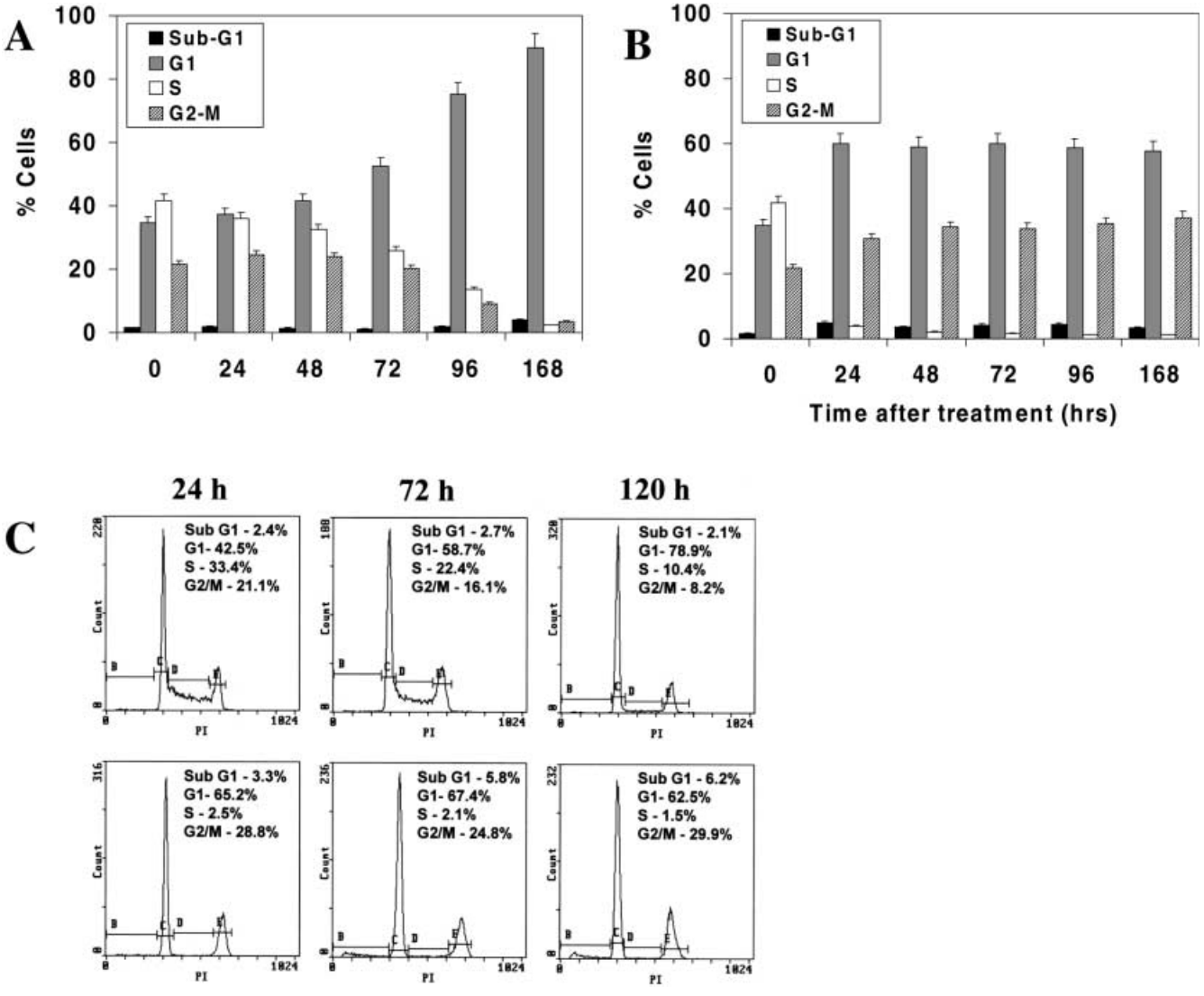

Fig. 5A–C Time-course cell cycle analysis after 2 h exposure to 1 μ*M* WMC26. **A**, **B** Cells used in the experiment the results of which are shown in Fig. 3A were fixed with 70% ethanol at various time-points, stained with propidium iodide and analyzed using a fluorescence-activated cell sorter (**A** untreated cells, **B** cells exposed for 2 h to 1 μ*M* WMC26). Cell percentages are averages of two separate experiments. **C** An example of typical FACS results from an independent experiment in which cells were untreated (*upper row*) or continuously exposed to 100 n*M* WMC26 (*lower row*)

more than 95% of cells were alive as evidenced by esterase activity and intact cellular membranes (Fig. 6D).

β-Galactosidase activity

HCT116 cells continuously exposed for 12 days to 100 n*M* WMC26 failed to show any cytochemically detectable β-galactosidase activity at pH 6. However, a significant difference was found between treated and untreated cells in the level of lysosomal β-galactosidase activity at pH 4 (Fig. 7). All treated cells showed a high level of β-galactosidase activity (Fig. 7A), which contrasted with the control cells, of which only a few cells showed weak activity of the enzyme (Fig. 7B).

Combined treatment with UCN-01

HCT116 cells which had been arrested with WMC26 for 12 days, were exposed to 1 μ*M* UCN-01 (Fig. 8). After 1 h of treatment a majority of the arrested cells dramatically changed morphology (Fig. 8B) and in 24 h about 70% of cells were dead (Fig. 8C), as evidenced by the MTT assay (data not shown). This characteristic change in cell morphology was not observed for exponentially growing control cells treated with the same concentration of UCN-01. To determine whether the WMC26-treated cells were sensitized to UCN-01, control cells and cells arrested with 100 n*M* WMC26 for 48 h were exposed for 120 h and 72 h, respectively, to UCN-01 and the cytotoxicity was measured by the MTT assay (Fig. 9). This experiment showed that 1 μ*M* UCN-01 was much more lethal to cells pretreated with WMC26. To find if the potentiation of UCN-01 cytotoxicity by

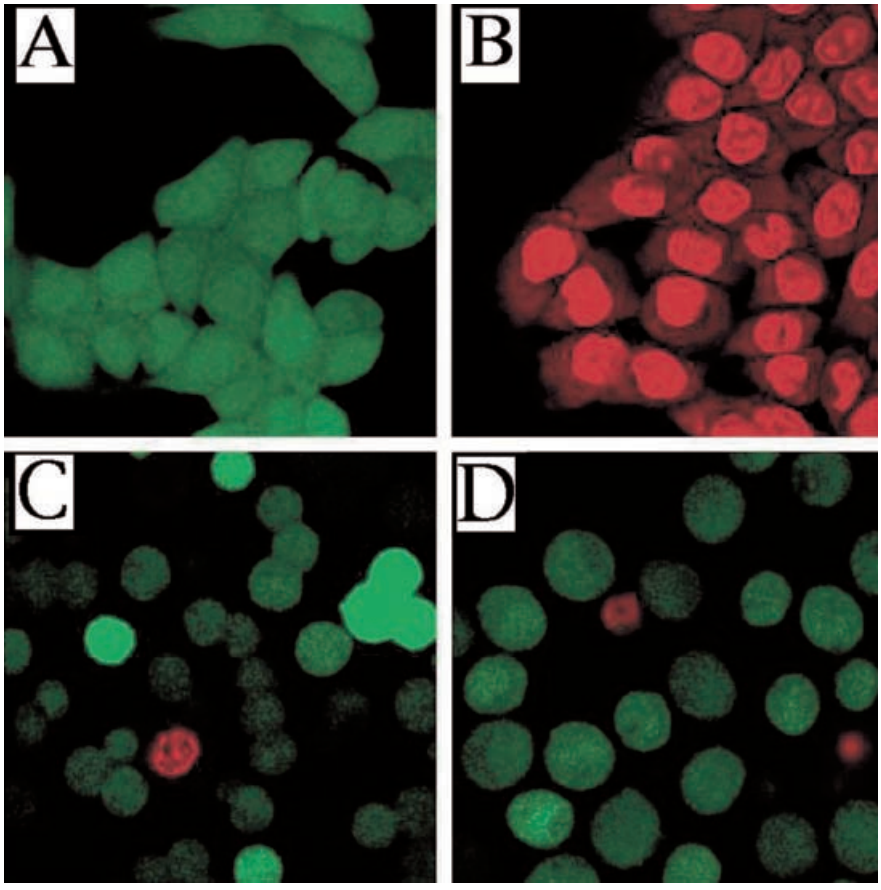

Fig. 6A–D The LIVE/DEAD two-color cell viability experiment (**A** untreated cells (LIVE), **B** cells treated with 70% ethanol for 30 min (DEAD), **C** cells exposed to 100 n*M* WMC26 for 12 days, **D** cells exposed to 1 μ*M* WMC26 for 12 days). Both attached and floating cells that were treated with WMC26 were included in the assay

WMC26 required initial pretreatment of the cells by WMC26, asynchronized cells were treated for 24 h with 100 n*M* WMC26, 1 μ*M* UCN-01 or with both drugs combined, and then analyzed by FACS for the presence of apoptotic cells (Fig. 10A). This experiment suggested enhancement of apoptosis by simultaneous addition of both drugs, indicating that concurrent treatment with WMC26 also potentiated the cytotoxic effect of UCN-01. The initiation of the apoptotic cascade was supported by the activation of caspase-3 [15], as indicated in Fig. 10B.

Discussion

Gel electrophoresis experiments, annexin V assay, examination of nuclei by confocal laser microscopy (data not shown) and flow cytometry measurements failed to detect any evidence of apoptotic processes in HCT116 cells treated with WMC26. Inspection of cells exposed to various concentrations of the drug revealed that at concentrations that inhibited colony formation, the vast majority of the initially seeded cells were still alive, as judged by trypan blue exclusion, after 2 weeks of incubation. Microscopic examination revealed significant morphological changes in treated cells. The cells were rounded and enlarged but remained attached. No changes typical of apoptosis were visible. Staining with DAPI did not show any fragmentation of the nucleus, although some chromatin condensation was evident (data not shown).

At concentrations of WMC26 of 100 n*M* and higher, exposures as short as 2 h were sufficient for complete cell growth inhibition after about 24 h of incubation. During that time almost complete depletion of the S-phase population occurred and cells became arrested at the G_1 and G_2/M phases. The growth arrest persisted for a long time and was not accompanied by cell death. Cells continuously exposed to 1 μ*M* and even 10 μ*M* WMC26 remained viable for 6 weeks and more.

The major finding that emerged from cell counting, MTT assays, FACS analysis, and LIVE/DEAD viability experiments was that WMC26 produces a strong cytostatic effect, but is not directly toxic to HCT116 cells. The fact that cells completely stopped DNA synthesis and lost their ability to divide but remained viable for a long time suggests a senescence-like state, which has been proposed for some drug-treated tumor cells [3, 30]. This prompted us to evaluate the activity of SA-*β*-Gal in treated cells. The activity of this enzyme, which is detected at pH 6, has been suggested to be a biomarker of senescence in normal somatic cells [6, 14, 27], and recently has been demonstrated in several tumor cell lines exposed to various drugs [3, 25, 30, 31]. SA-*β*-Gal is different from lysosomal *β*-galactosidase, which has an optimum activity at pH 4. Our experiments failed to

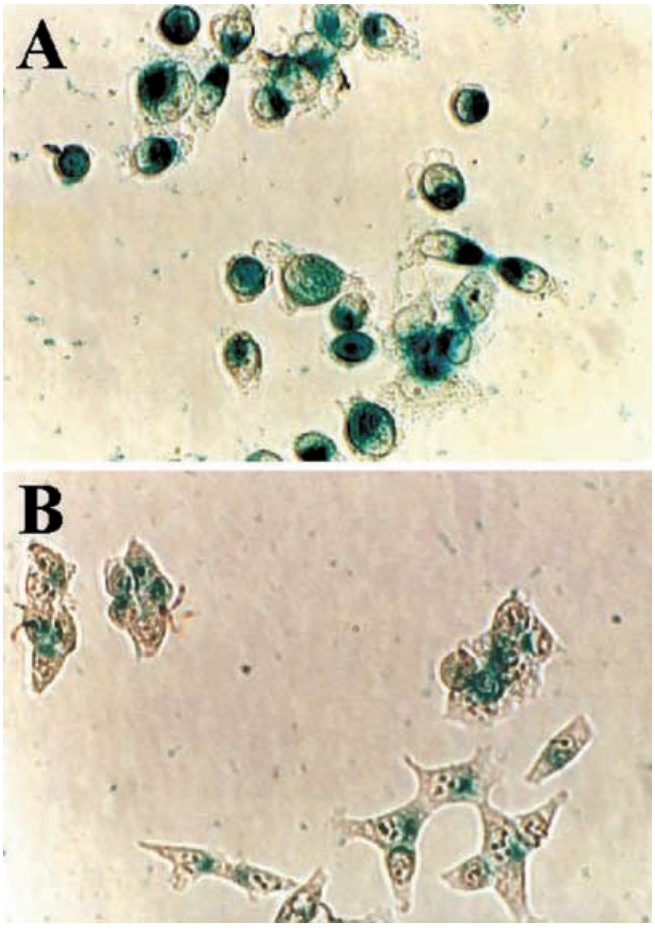

Fig. 7A, B The induction of β-galactosidase in HCT116 cells exposed to WMC26. Cells were incubated with 100 n*M* of the drug for 12 days, then fixed and stained for β-galactosidase at pH 6 and pH 4. No β-galactosidase staining was visible at pH 6. **A** Representative photograph of β-galactosidase activity at pH 4 in treated cells taken at ×200 magnification. **B** Staining of untreated control was marginal under the same conditions

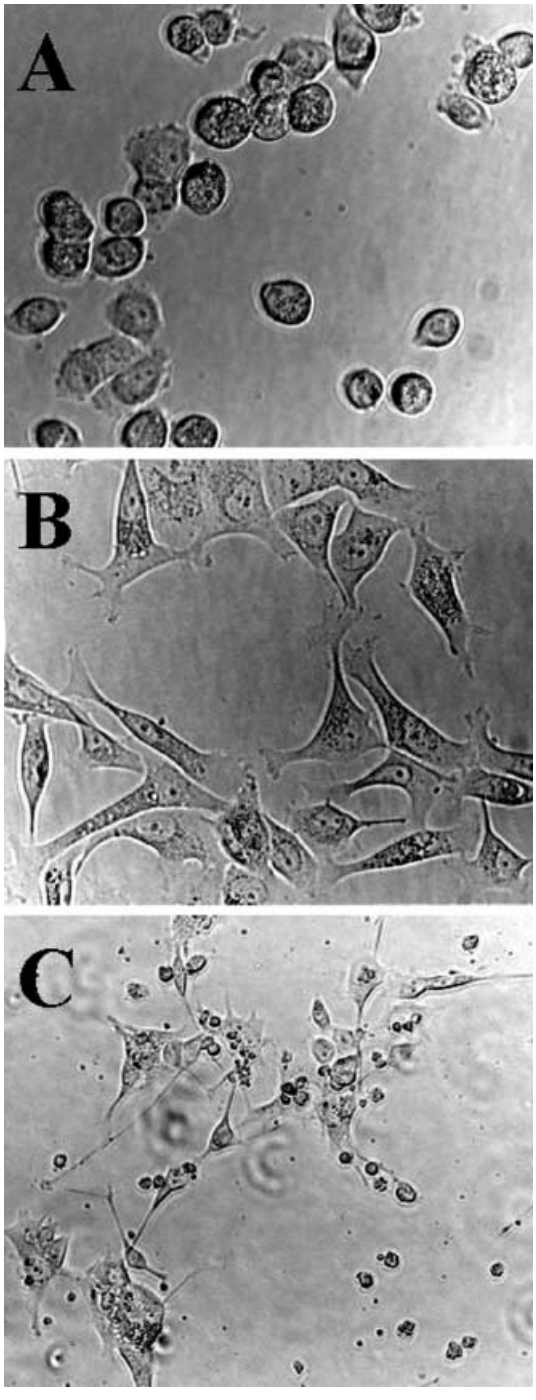

Fig. 8 Effect of UCN-01 on WMC26 growth-arrested HCT116 cells (**A** cells exposed continuously to 0.1 μ*M* WMC26 for 12 days, **B** the same cells after 1 h treatment with 1 μ*M* UCN-01, **C** 24 h after treatment with UCN-01 most cells are either dead or dying)

detect any SA-β-Gal at pH 6 in HCT116 cells treated with WMC26. However, we did observe a significant difference between treated and untreated cells in the level of lysosomal β-galactosidase activity at pH 4. Recently, Chang et al. [3] have reported enhanced expression of SA-β-Gal in HCT116 cells treated with doxorubicin. We confirmed the activity of SA-β-Gal at pH 6 in these cells treated with doxorubicin but not in cells treated with WMC26. These results suggest that the growth-arrested cells produced by WMC26 treatment are different from the senescence-like phenotype of HCT116 induced by doxorubicin treatment. Experiments on the cellular localization of WMC26 (L. Hernandez, unpublished results) have shown that WMC26 eventually becomes localized in lysosomes. Thus, the upregulation of lysosomal β-galactosidase activity may be triggered by the high concentration of the drug in that organelle.

The unique feature of WMC26 is the selective and *complete* cell growth inhibition observed at relatively low concentrations. This agent arrests the entire cell population at the G_1 and G_2/M checkpoints, most probably as a result of a specific DNA interaction. It was reasonable to expect then that sequential treatment of the growth-arrested cells with abrogators of cell cycle checkpoints would result in selective cell killing. We found that UCN-01, the best-known cyclin-dependent kinase inhibitor and G_2/M-checkpoint abrogator [29, 32], was able to override the WMC26-induced cell

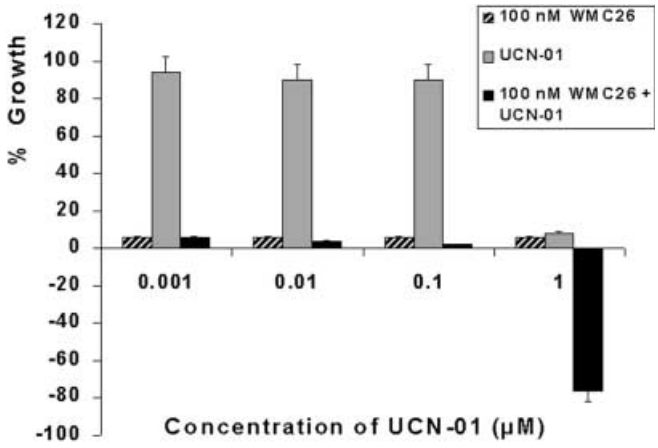

Fig. 9 Potentiation of UCN-01 cytotoxic effect in HCT116 cells pretreated with WMC26. Cells pretreated for 48 h with 100 n*M* WMC26 were exposed for 72 h to UCN-01 and their viability was determined by the MTT cell proliferation assay. Control cells were treated with UCN-01 for 120 h. Results are presented as percent growth: $100 \times (T - T_0)/(C - T_0)$, where T_0, T, and C are the absorbance values at 540 nm at time 0, and in treated and control cells, respectively, and are the means of three independent experiments

growth arrest. The abrogation of growth arrest caused a massive cell killing, suggesting that the cells were sensitized to induction of apoptosis by UCN-01. In addition, we showed that the potentiation of apoptosis was independent of whether the exposure to UCN-01 was sequential or simultaneous with WMC26 treatment.

The use of UCN-01 as a component of drug combination has been studied previously for such DNA-damaging agents as mitomycin C [1, 24], cisplatin [2, 10,

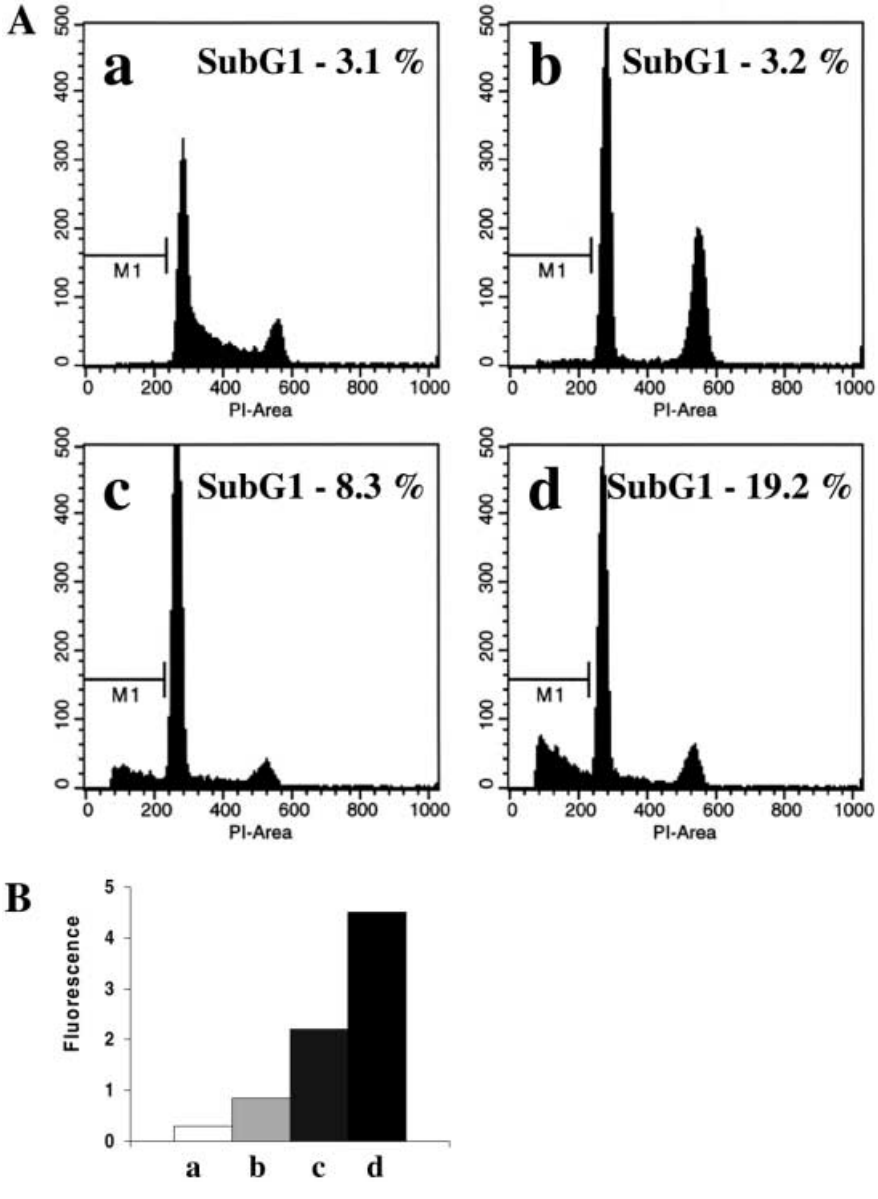

Fig. 10A, B Induction of apoptosis in HCT116 cells evaluated by FACS analysis (**A**) and caspase-3 activation (**B**) after 24 h treatment with a single agent or a combination as follows: *a* control cells, *b* 100 n*M* WMC26, *c* 1 μ*M* UCN-01, *d* a mixture of 100 n*M* WMC26 and 1 μ*M* UCN-01

13, 29], γ-irradiation [29] and camptothecin [11, 20]. In all these studies UCN-01 was used to enhance the cytotoxicity caused by a DNA-damaging agent. However, UCN-01 itself has been demonstrated to have cytotoxic effects in many different cancer cells [8, 16, 21, 28]. It is currently being tested as a single agent in clinical trials, based on its in vitro growth inhibitory activity as well as its antitumor activity in animal models [17]. The data presented here demonstrate for the first time that the cytotoxic activity of UCN-01 can be significantly enhanced by combination with a cytostatic agent such as WMC26. An attractive hypothesis for the combined activity of WMC26 and UCN-01 is that the latter agent forces the WMC26-arrested cells into premature completion of the cell cycle, which results in the triggering of the apoptotic cascade as evidenced by activation of caspase-3 (Fig. 10B). Our findings suggest that the highly selective BIAs may be useful in combination therapies together with cyclin-dependent kinase inhibitors [19, 22, 23]. UCN-01 was required at supraphar-macological concentrations to produce this effect. However, preliminary data, which support the generality of this hypothesis, indicate that other cyclin-dependent kinase inhibitors such as flavopiridol are effective at much lower concentrations.

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