The Apoptotic Effects and Synergistic Interaction of Sodium Butyrate and MG132 in Human Retinoblastoma Y79 Cells

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ABSTRACT

This study deals with the apoptotic effect exerted on human retinoblastoma Y79 cells by both sodium butyrate and an inhibitor of 26S proteasome [z-Leu-Leu-Leu-CHO (MG132)] and their synergistic effect. Exposure to sodium butyrate (1–4 mM) induced an accumulation of cells in the G2-M phase that was already visible after 24 h of treatment, when morphological and biochemical signs of apoptosis appeared only in a small number of cells (5–10%). Thereafter, the apoptotic effects increased progressively with slow kinetics, reaching a maximum after 72 h of exposure, when they concerned a large fraction of cells (>75% with 4 mM sodium butyrate). Sodium butyrate stimulated the conversion of procaspase-3 into caspase-3 and also induced the cleavage of poly-(ADP-ribose) polymerase and lamin B, two hallmarks of apoptosis. All of the apoptotic signals were suppressed by benzyloxy carbonyl-Val-Ala-Asp-fluoromethylketone (a general inhibitor of caspase activities), whereas acetyl-Asp-Glu-Val-Asp aldehyde, a specific inhibitor of caspase-3 activity, only induced a partial reversion of the apoptotic effects. Sodium butyrate also decreased the Bcl-2 level, whereas it increased the Bax level and stimulated the release of cytochrome c from the mitochondria, an event that was most likely responsible for the activation of caspase-3. Finally, sodium butyrate activated 26S proteasome, the major extralysosomal degradative machinery, which is responsible for the degradation of short-lived proteins. Consequently, the levels of p53, N-myc, and IκBα (factors that play regulatory roles in apoptosis) diminished, whereas the nuclear level of nuclear factor κB concomitantly increased. Treatment of Y79 cells with MG132 induced apoptosis with more rapid kinetics than with sodium butyrate. The effects appeared after 8 h of incubation, reaching a maximum at 24 h, and they were accompanied by increased levels of N-myc, p53, and IκBα. MG132 also favored the release of cytochrome c from the mitochondria and increased the activity of caspase-3. When Y79 cells were exposed to combinations of sodium butyrate and MG132, the latter compound suppressed the decreasing effect induced by sodium butyrate on the levels of p53, N-myc, and IκBα and the increasing effect on the nuclear level of nuclear factor κB. Moreover, an increase in the level of Bax and an enhancement in the release of cytochrome c from the mitochondria were observed. Clear synergistic effects concerning the activation of both caspase-3 and apoptosis were induced by a combination of suboptimal doses of sodium butyrate and MG132. The results support the conclusion that MG132 potentiates the apoptotic effect of sodium butyrate by suppressing its stimulatory effect on 26S proteasome activity. Synergistic interactions between butyrate and inhibitors of proteasome could represent a new important tool in tumor therapy and, in particular, the treatment of retinoblastoma.

INTRODUCTION

Butyrate, a short-chain fatty acid, is physiologically produced by the bacterial fermentation of dietary fibers in the human colon. It is used by colorectal cells as the most important source in the production of energy (1). Moreover, butyrate inhibits in vitro cell growth by favoring cell cycle arrest and promotes differentiation in normal as well as transformed cells (2). Besides these effects, sodium butyrate induces apoptosis in a number of cancer cells (3–6). The differentiation and apoptotic actions of butyrate could partially explain why this compound exerts a role in the prevention of colorectal cancer (3).

Despite the fact that the antiproliferative and apoptotic effects of butyrate have been studied by many authors, the mechanism of its action remains unclear. Because sodium butyrate inhibits histone deacetylase, it seems probable that the consequent histone hyperacetylation may lead to chromatin relaxation and altered gene expression (7). To this end, Medina et al. (4) have observed that sodium butyrate and trichostatin A, another inhibitor of histone deacetylase, induce apoptosis in Jurkat lymphoid and colorectal cancer cell lines, favoring the conversion of the proenzyme form of caspase-3 to the catalytically active enzyme. Moreover, they also suggest that butyrate leads to the expression of a protein that facilitates the pathway by which the mitochondria activate caspase-3. Other authors have demonstrated that butyrate decreases the level of p53 in transformed cells and induces apoptosis in a manner that is independent of p53, a protein correlated with apoptosis in many cells and tissues (5). Finally, it has been ascertained (6) that in colon cell lines, butyrate potentiates Fas-dependent apoptosis induced by the exposure of the cells to Fas ligand.

During recent years, we have carried out research on Y79 human retinoblastoma cells, a cell line that lacks pRB, due to the inactivation of both alleles of the tumor suppressor gene RB-1 (8). Loss of pRb occurs in retinoblastoma and osteosarcoma and also appears to contribute to the progression of many other malignancies (9). In its hypophosphorylated state, pRb sequesters and inactivates transcription factors of the E2F family, which are required for the activation of S-phase genes. Phosphorylation of pRb results in the release of E2F factors, allowing cells to enter the cell cycle (10). Another interesting function of pRb is its capacity to suppress apoptosis (11, 12). This has been established by the finding that pRb-deficient cells seem to be more susceptible to apoptosis than cells with a functional RB gene (13). In pRb-deficient cells, active E2F can be responsible for progression through the cell cycle or, in some circumstances, for the induction of apoptosis (14). There is also increasing evidence that p53 is required for much of the apoptosis seen in pRb-deficient cells, and that p53 and pRb may have opposing roles in the control of apoptosis (12).

A number of authors have already studied the effect of sodium butyrate on Y79 cells in vitro. Kyritsis et al. (15) have observed a marked inhibition in growth together with the appearance of an elongated form of cells. Successively, Conway et al. (16) demonstrated that treatment with sodium butyrate efficaciously stimulated apoptosis in Y79 cells and induced fragmentation of DNA. We have

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4 The abbreviations used are: pRB, retinoblastoma protein; AO/EB, acridine orange/ethidium bromide; Ac-DEVD, acetyl-Asp-Glu-Val-Asp aldehyde; MTT, 3-(4,5-dimeth-ylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; MG132, z-Leu-Leu-Leu-CHO; PARP, poly-(ADP-ribose) polymerase; z-VAD-fmk, benzyloxy carbonyl-Val-Ala-Asp-fluoromethylketone; NFκB, nuclear factor κB. 5586

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have undertaken a research project on programmed cell death in \( \text{Y79} \) cells and have individuated a number of compounds that are capable of triggering apoptosis (17–20). Many of these compounds also increased the level of p53.

This study deals with research on the apoptotic mechanism triggered by sodium butyrate in \( \text{Y79} \) cells. We demonstrate that butyrate induced apoptosis with slow kinetics in these cells. At the same time, butyrate stimulated the activity of 26S proteasome, with a consequent decrease in the level of short-lived proteins such as p53, N-myc, and \( \text{IxB} \), which have a role in apoptosis. On the other hand, apoptosis in \( \text{Y79} \) cells was also induced by MG132, an inhibitor of 26S proteasome. Interestingly, MG132 suppressed the effect of butyrate on p53 and the other short-lived proteins and synergized with butyrate in the induction of apoptosis.

**MATERIALS AND METHODS**

**Cell Culture and Reagents.** Human retinoblastoma \( \text{Y79} \) cells were grown in suspension in RPMI 1640 and 10% FCS as described previously (20). For monolayer cultures, cells were usually seeded at 1.5 \( \times \) 10^5 cells/cm² on 96-well culture plates precoated with poly-d-lysine (\( M_\text{s} 150,000–300,000; 5 \mu g/cm² \)); allowed to equilibrate for 24 h in serum-free DMEM-F-12 medium (2:1; \( v/v \)) supplemented with transferrin (5 \( \mu g/ml \)), sodium selenite (5 \( \mu g/ml \)), 10 mm HEPES, and 28 mm NaHCO\(_3\) (pH 7.4); and treated with chemicals or vehicle (control cells) as described in the figure legends and text. Cell viability was determined by the MTT colorimetric assay (21) as described previously(22).

\( z\)-VAD-fmk was obtained from Bachem AG (Bubendorf, Switzerland). Ac-DEVD was obtained from Sigma (St. Louis, MO), and MG132 was obtained from Biomol Research Laboratories, Inc. (Plymouth Meeting, PA). Stock solutions of \( z\)-VAD-fmk (100 mm) were prepared in methanol, and stock solutions of both Ac-DEVD (200 mm) and MG132 (50 mm) were prepared in DMSO.

**Determination of Apoptosis.** Morphological evidence of apoptosis was obtained by means of AO/EB staining. Monolayer cultures in 96-well plates were used for these studies. After removal of the incubation medium, cells were rinsed and treated with a solution composed of AO/EB (100 \( \mu g/ml \) PBS of each dye). Cells were examined by fluorescence microscopy and photographed (ASA 1000 film; Kodak, Rochester, NY). Viable cells appear green with intact nuclei. Nonviable cells have bright orange chromatin. Apoptosis was demonstrated by the appearance of cell shrinkage with condensation and fragmentation of nuclei. Apoptotic cells were easily distinguished from necrotic cells because the latter appeared orange with a normal nuclear structure. This procedure was used to quantify the number of apoptotic cells after treatment with sodium butyrate or MG132. Apoptosis was also quantified using an ELISA kit (Boehringer Mannheim) that measured the amounts of mono- and oligonucleosomes produced in the cytoplasmic fraction of lysed cells as a consequence of DNA fragmentation (18). After treatment, cells were collected and lysed as reported in the manufacturer’s instructions. After centrifugation at 20,000 \( \times \) \( g \) for 15 min, equal amounts of 0.3 \( \mu g \) of protein) of cytosolic fractions were used for the assays.

**Cell Cycle Distribution using Flow Cytometry.** The distribution of cells in the cell cycle phases was determined as reported by Carollo et al. (23). Briefly, at the end of the incubation, \( \text{Y79} \) cells were washed twice with ice-cold PBS and resuspended at 1 \( \times \) 10^5 cells/ml in a hypotonic fluorochrome solution containing 50 \( \mu g/ml \) propidium iodide in 0.1% sodium citrate plus 0.03% (\( v/v \)) NP40. After 30 min of incubation, the samples were filtered through 40-\( \mu \)m nylon mesh cloth, and fluorescence was analyzed as a single-parameter frequency histogram. Data were analyzed using ModFit TL software (Becton Dickinson).

**Western Blot Analysis.** Cells were lysed for 30 min at 4°C with a solution (15 \( \mu l/10^6 \) cells) composed of 1% NP40, 0.5% sodium deoxycholate, and 0.1% SDS in PBS (pH 7.4) and sonicated three times for 10 s in a Soniprep 150 apparatus (MSE). The following inhibitors of proteases were added: (a) 25 \( \mu g/ml \) phenylmethylsulfonyl fluoride; (b) 1 mm sodium orthovanadate; (c) 10 mm Na\(_3\)VO\(_4\); (d) 10 mm Na\(_2\)PO\(_4\); (e) 25 \( \mu g/ml \) leupeptin; and (f) 0.2 mm sodium PP\(_3\). Cell lysates were centrifuged at 15,000 \( \times \) \( g \) for 20 min at 4°C. Equivalent amounts of protein (60 \( \mu g \)) were resolved by 10% SDS-PAGE and transferred onto nitrocellulose for detection with antibodies. The primary antibodies used were: (a) goat polyclonal antihuman Bax (1:500); (b) CPP32 (1:1000); (c) lamin B (M-20; 1:1000); (d) rabbit polyclonal antihuman PARP (1:2000); (e) mouse monoclonal antihuman Bcl-2 (1:1000); (f) p53 (1:1000); (g) p21 (187; 1:500), (h) N-myc (1:1000); and (i) EFZ (1:1000). All antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Visualization was performed using nitroblue tetrazolium and bromochloro-indolyl-phosphate.

To evaluate the amount of Nf\( \text{EB} \) and I\( \text{xB} \), nuclear and cytoplasmic extracts were prepared as described previously by Roff et al. (24). For immuno blot analysis, equal amounts of nuclear and cytoplasmic extracts (60 \( \mu g \)) were resolved by SDS-PAGE (10%) and immunoblotted using rabbit polyclonal antihuman Nf\( \text{EB} \) (1:1000) or I\( \text{xB} \) (C-15; 1:1000) antibodies (Santa Cruz, Biotechnology). Finally, bands were visualized using the enhanced chemiluminescence system (Amersham) and quantified by densitometric analysis.

**In Vitro Assay of Cytochrome \( \text{c} \) Efflux From the Mitochondria.** Subcellular fractions were prepared as described by Yang et al. (25). Briefly, cells (5 \( \times \) 10^5) were washed twice in PBS and resuspended in lysis buffer (20 mm HEPES-KOH (pH 7.5), 10 mm KCl, 1.5 mm MgCl\(_2\), 1 mm EDTA, 1 mm DTT, and 0.1 mm phenylmethylsulfonyl fluoride) containing 250 mm sucrose. Cells were homogenized with 20 strokes of a Teflon homogenizer, and the homogenates were centrifuged at 750 \( \times \) \( g \) for 10 min at 4°C. The resulting supernatants were centrifuged at 10,000 \( \times \) \( g \) for 30 min. The mitochondria pellets were resuspended in the same buffer. The cytosolic fraction was obtained after centrifugation at 100,000 \( \times \) \( g \) for 1 h at 4°C. For immunoblot analysis, equal amounts of proteins (50 \( \mu g \)) were subjected to 10% SDS-PAGE. After transferring to nitrocellulose filter, the filter was probed by mouse monoclonal antihuman cytochrome \( \text{c} \) antibody (1:1000).

**In Vitro Assay of Caspase-3 Activity.** \( \text{Y79} \) cells were lysed for 1 h as reported in the manufacturer’s instructions and centrifuged at 15,000 \( \times \) \( g \) for 15 min at 4°C. Assays were performed in 96-well plates using the ApoAlert CPP32 colorimetric assay (Clontech Laboratories Inc., Palo Alto, CA), which is based on spectrophotometric detection of the chromophore p-nitroanilide after cleavage from the labeled substrate of the enzyme, DEVD-p-nitroanilide. The manufacturer’s instructions were followed.

**Assay of 26S Proteasome Activity.** \( \text{Y79} \) cells were lysed as reported by Beyette et al. (26) using ice-cold homogenization buffer (20 mm Tris-HCl (pH 7.2), 0.1 mm EDTA, 1 mm 2-mercaptoethanol, 5 mm ATP, 20% (\( v/v \)) glycerol, and 0.04% NP40). The lysates were centrifuged at 13,000 \( \times \) \( g \) at 4°C for 15 min to remove insoluble material. The high-speed supernatants were assayed for 26S proteasome activity following the indications of Rivett et al. (27). Briefly, assay mixtures containing 50 mm HEPES/KOH (pH 7.5) and 40 \( \mu l \) N-succinyl-Leu-Leu-Val-Tyr-7-amido-4-methylcoumarin as substrate and the supernatants were prepared in a total volume of 200 \( \mu l \) and incubated at 37°C for 30 min. The reaction was stopped by the addition of 0.1 ml of stop mix (0.3 mm acetate trihydrate/ acetic acid). Fluorescence (excitation, 370 nm; emission, 430 nm) was measured by using a luminescence spectrometer (Perkin-Elmer LS50).

**RESULTS**

**The Effects of Sodium Butyrate on Viability and Apoptosis in Human Retinoblastoma \( \text{Y79} \) Cells.** Our study was performed using \( \text{Y79} \) cells grown as serum-free monolayer cultures. We have observed by means of light microscopy (data not shown) that treatment with sodium butyrate (2 mm) initially produced clusters of packed cells. Successively, during the second day of treatment, a number of cells exhibited a flattened polygonal morphology, whereas other cells showed morphological features of apoptosis, consisting of a decrease in cell volume, membrane blebbing, and nucleus fragmentation. After 72 h of treatment, the bulk of cells appeared seriously damaged.

To investigate the type of cell death induced by sodium butyrate, cells were stained with AO/EB, which allows the identification of viable, apoptotic, and necrotic cells based on color and appearance. Staining with AO/EB of the samples treated for 3 days with 2 mm sodium butyrate (Fig. 1B) showed a large percentage (about 45%) of
orange-stained cells. Moreover, the majority of these nonviable cells, together with about 40% of the remaining viable cells, showed fragmented nuclei and other signs of apoptosis.

Treatment of Y79 cells with sodium butyrate reduced the number of viable cells measured with MTT in a dose- and time-dependent manner (Fig. 2A). With 2 mM sodium butyrate, a clear effect was already observed at 24 h of treatment (~21%), after which cell viability diminished progressively; after 72 h, the number of cells was reduced by about 70%. The apoptotic effect exerted by sodium butyrate was quantified by counting the frequency of nonviable cells with fragmented nuclei in samples stained with AO/EB. As shown in Fig. 2B, sodium butyrate induced apoptosis with slower kinetics than other agents (17–20), so that at 24 h of treatment with 2 mM sodium butyrate, only about 5% of the cells showed apoptotic signs. On increasing the time of treatment, this percentage increased progressively up to 72 h, when about 45% of cells were apoptotic. The effect was also dose dependent, with the maximum (75–80% of apoptotic cells) observed after 72 h with 4 mM sodium butyrate (Fig. 2B).

Flow cytometric analysis showed that in Y79 cells, sodium butyrate induced an arrest of the cell cycle at the G2-M phase. The effect, which was already evident at 24 h of treatment, increased with time, so that at 48 h in the presence of 2 mM sodium butyrate, about 35% of cells were arrested at the G2-M phase, whereas the subdiploid population (apoptotic cells) corresponded to about 18% (Fig. 3). Moreover, as previously demonstrated by Conway et al. (16), sodium butyrate induced oligonucleosomal fragmentation of DNA in Y79 cells with the appearance of the ladder pattern of DNA in agarose gel electrophoresis (data not shown).

The degree of apoptosis induced by sodium butyrate was also estimated by means of an ELISA kit, which measures the quantity of nucleosomal fragments in apoptotic cells. The results (data not shown) confirmed that sodium butyrate induced apoptosis with a dose- and time-dependent effect. The maximum enrichment in the level of DNA fragmentation (an 8-fold increase) was found after treatment with 4 mM sodium butyrate for 3 days.

We studied the influence of z-VAD-fmk and Ac-DEVD, two membrane-permeable inhibitors of caspase activity, on the effect exerted by sodium butyrate on Y79 cells. z-VAD-fmk, a general inhibitor of caspases, counteracted the apoptotic effects induced in these cells by sodium butyrate (Fig. 2C) and at the same time reduced the degree of DNA fragmentation (data not shown). The effect was time and dose dependent, with the apoptotic signs being completely abolished by 100 μM z-VAD-fmk. This fact strongly suggested that caspase activities are involved in the apoptotic mechanism triggered by butyrate in Y79 cells. Nevertheless, the addition of z-VAD-fmk was not capable of completely suppressing the effect exerted by sodium butyrate on cell viability. In fact, when 100 μM z-VAD-fmk was added to 2 mM sodium butyrate, the number of cells still diminished, although only by 30% at 72 h (Fig. 2A). Ac-DEVD, a selective inhibitor of caspase-3, also decreased the apoptotic effect induced by sodium butyrate. However, in contrast to z-VAD-fmk, Ac-DEVD was not capable, even at a dose of 800 μM, of entirely suppressing apoptosis, which was still observed in about 24% of cells (Fig. 2D).

The Effects of Sodium Butyrate on the Activities of Caspases and 26S Proteasome. Caspase-3, a cysteine protease, is present in cells as an inactive proenzyme. Activation of this form requires cleavage at specific aspartate sites to produce subunits of M, 17,000 and M, 12,000. The active enzyme takes part in the execution phase of apoptosis (28).

Our experiments have demonstrated that sodium butyrate induced the activation of caspase-3 in Y79 cells. The direct estimation of caspase-3 activity showed a 2-fold increase after exposure to 2 mM sodium butyrate for 24 h and a 3-fold increase after 48 h of treatment (Fig. 4A). The effect was inhibited by both z-VAD-fmk and Ac-DEVD (Fig. 4A). The activation of caspase-3 was also shown by means of Western blotting analysis, using a primary antibody that recognizes both the M, 32,000 proenzyme and the M, 12,000 subunit of the active enzyme. As shown in Fig. 5, in the cells treated with 2 mM sodium butyrate, the p12 subunit appeared at 24 h of exposure. Thereafter, its level increased up to 72 h of treatment, whereas that of the proenzyme diminished. The addition of z-VAD-fmk suppressed the production of the p12 subunit induced by sodium butyrate, providing evidence that the activation of caspase-3 was stimulated by caspase activities inhibited by z-VAD-fmk.

To demonstrate the proteolytic activity of caspases in cells treated with sodium butyrate, we studied the fragmentation of two nuclear proteins, PARP and lamin B, which are cleaved by caspase activities (29). PARP is a protein of M, 116,000 that is associated with chromatin and is cleaved in a number of cell death systems by cysteine proteases to yield two fragments of M, 85,000 and M, 24,000. Western blotting analysis revealed that treatment of Y79 cells with sodium butyrate induced the proteolytic cleavage of M, 116,000 PARP protein

Fig. 1. Identification of apoptotic cells by AO/EB staining and the effect induced by sodium butyrate and MG132 and the influence of z-VAD-fmk. Y79 cells were treated with 2 mM sodium butyrate for 72 h (B and C) or with 2 μM MG132 for 24 h (D and E) in the absence (B and D) or presence (C and E) of 100 μM z-VAD-fmk and compared with untreated cells (A). The most representative fields are shown.
to yield the characteristic $M_r 85,000$ fragment. With 2 mM sodium butyrate (Fig. 5), the effect was already evident at 24 h of treatment, whereas at 3 days, PARP was almost entirely cleaved. Fig. 5 also shows that sodium butyrate induced the cleavage of lamin B. The effects on both the cleavage of PARP and lamin B were suppressed by the addition of $z$-VAD-fmk.

As shown in Fig. 6A, the activity of 26S proteasome measured in high-speed supernatants increased with time in cells treated with 2 mM sodium butyrate, reaching a value 3.2 times higher than the control after 48 h of exposure. Moreover, the addition of either 0.2 mM or 0.5 mM MG132 completely suppressed the effects induced on proteasome activity by treatment for 24 h with 0.5 and 2 mM sodium butyrate, respectively (Fig. 6B).

The Effects of the Inhibitor of 26S Proteasome MG132 on Apoptosis and Caspase-3 Activity in Y79 Cells. MG132 is a peptide aldehyde that acts as a potent competitive inhibitor of the chymotrypsin-like activity of the proteasome complex (30). Our results clearly demonstrate that treatment with MG132 also induced...
apoptotic involution in Y79 cells (Fig. 1D). The effect, which was dose and time dependent (Fig. 2F), appeared earlier in cells treated with MG132 than in cells treated with sodium butyrate. In fact, a large percentage of apoptotic cells (about 40%) was already observed at 8 h of incubation with 2 μM MG132. The maximum effect was reached at 24 h, when about 80% of cells showed signs of apoptosis. Concomitantly with the increase in the degree of apoptosis, the number of cells decreased with time of treatment (Fig. 2E). Furthermore, exposure of Y79 cells to MG132 enhanced the activity of caspase-3 (Fig. 4B), which became 7 times higher than that of the control when the cells were treated with 2 μM MG132 for 12 h. z-VAD-fmk (100 μM) was capable of suppressing the effects exerted by MG132 on both apoptosis (Figs. 1E and 2F) and caspase activity (Fig. 4B).

The Effect of Sodium Butyrate and MG132 on the Level of p53, p21, N-myc, and E2F. The tumor suppressor protein p53 has a short half-life. It is well known that the ubiquitin-proteasome system plays an important role in the degradation of many short-lived proteins, including p53 (31). As shown in Fig. 7A, treatment of Y79 cells with sodium butyrate lowered the level of p53. With 2 mM sodium butyrate, the p53 level decreased by 40% at 24 h with 0.5 mM sodium butyrate. In contrast, the maximum was found at 1–2 μM MG132 (Fig. 8), whereas the maximum was found at 1–2 μM MG132. N-myc, a short lived protein that behaves as a transcriptional factor, is completely cleaved by the ubiquitin-proteasome system (32). N-myc is implicated in the control of cell proliferation and in the...
induction of apoptosis (33) and is overexpressed in human retinoblastoma cells (34). As we have demonstrated for p53, treatment with sodium butyrate (2 mM) decreased the level of N-myc, with a maximum effect after 72 h of incubation (Fig. 7A). On the contrary, treatment with MG132 in the range included between 0.2 and 2 µM resulted in a dose-dependent increase in N-myc. The effect was rapid and reached its maximum at 16 h of exposure, when the level of N-myc became two to three times higher than the controls in the presence of 2 µM MG132 (Fig. 7B).

Interestingly, when Y79 cells were treated with combinations of MG132 and sodium butyrate, the inhibitor of proteasome counteracted the decreasing effects of butyrate on both p53 and N-myc. Treatments with 0.2 or 0.5 µM MG132 prevented both p53 and N-myc from the lowering effects induced by 0.5 and 2 mM butyrate, respectively. In this way, in cells exposed for 24 h to combined treatments, the levels of p53 and N-myc were higher in both cases than those in the control and were identical to the levels found in cells treated with MG132 alone (Fig. 8).

It is well known that p21/Waf1, a transcriptional target of p53, plays a role in the control of the cell cycle. Moreover, it has been reported that sodium butyrate is capable of stimulating p21/Waf1 expression in a p53-independent manner (35). Our results demonstrate that in Y79 cells, butyrate increased the level of p21 protein. With 2 mM sodium butyrate, the effect appeared after 24 h of treatment and reached the maximum at 72 h (Fig. 7A). MG132 did not exert any effect on the level of p21 (Fig. 7B).

E2Fs are transcriptional factors that play a key regulatory role in cell cycle progression and seem to be involved in apoptosis (14). Moreover, E2Fs have been considered as a target of the ubiquitin-proteasome system (36). Our results demonstrate that treatment of Y79 cells with sodium butyrate (2 mM) decreased the level of E2F-1 in a time-dependent manner (Fig. 7A). The addition of MG132 (2 µM) also lowered the level of E2F-1 (Fig. 7B). Moreover, when MG132 (2 µM) was added to sodium butyrate (2 mM), the level of E2F-1 showed a further decrease (data not shown). Finally, treatment with both sodium butyrate and MG132 was unable to modify the level of other components of the E2F family (E2F-2 and E2F-4).

The Effects of Sodium Butyrate and MG132 on the Level of NFκB and Its Inhibitor, IκBα. In unstimulated cells, the transcriptional factor NFκB is sequestered in the cytoplasm as inactive complexes together with the inhibitory IκB proteins (37). The activation of NFκB depends on the phosphorylation of IκBα protein and the

![Fig. 7](image-url) Western blotting analysis of p53, p21, N-myc, and E2F-1 in Y79 cells treated for the indicated times with 2 mM sodium butyrate (A) or 2 µM MG132 (B). Cell lysates were resolved by SDS-PAGE, and proteins were immunoblotted and detected using specific antibodies.

![Fig. 8](image-url) The effects of combinations of sodium butyrate and MG132 on the levels of p53, N-myc, Bcl-2, Bax, and mitochondrial and cytosolic cytochrome c in Y79 cells. Cells were treated for 24 h with sodium butyrate or MG132 used separately or in combination as indicated in the figure. Cell lysates were resolved by SDS-PAGE, and proteins were immunoblotted and detected using specific antibodies.
Sodium Butyrate and MG132. The degree of apoptosis was estimated as reported in "Materials and Methods," resolved by SDS-PAGE, immunoblotted, and detected using specific antibodies.

consequent degradation of this inhibitory factor by 26S proteasome. As a result of the loss of IκBα, NFκB translocates into the nucleus and activates some responsive genes.

We have demonstrated by means of Western blotting analysis that sodium butyrate lowered the cytosolic level of IκBα in a dose- and time-dependent manner and induced a concomitant increase in the nuclear level of the p50 NFκB subunit. The greatest effects were found by treating the cells for 48 h with 2 mM butyrate (Fig. 9, A and B), although the effects also appeared clearly with 0.5 mM sodium butyrate (data not shown). Analysis of NFκB in the cytosol also revealed that its p105 precursor decreased with time of treatment, reaching much lower values 48 h after the addition of sodium butyrate (Fig. 9C). Moreover, the addition of 0.5 μM MG132 to 2 mM butyrate completely prevented cytosolic degradation of IκBα and nuclear accumulation of NFκB during the 24 h of treatment (Fig. 9, A and B). Similar results were obtained when 0.2 μM MG132 was added to 0.5 mM butyrate (data not shown). These results suggested that activation of 26S proteasome by butyrate induced the proteolytic cleavage of both IκBα and p105 and the consequent translocation of NFκB into the nucleus.

The Mitochondria Are Involved in the Apoptosis Induced by Sodium Butyrate and MG132. Recent evidence indicates that the mitochondria play a decisive role in apoptosis (38), functioning as integrators of different proapoptotic signaling pathways. In response to these signals, the mitochondria activate megachannels (also called permeability transition pores) that are present between the inner and outer mitochondrial membranes. Bcl-2, a fundamental death antago-

Fig. 9. Western blotting analysis of IκBα and NFκB in Y79 cells treated for the indicated times with 2 mM sodium butyrate in the absence or presence of 0.5 μM MG132. Cytoplasmic (A and C) and nuclear (B) extracts were prepared as reported in "Materials and Methods," resolved by SDS-PAGE, immunoblotted, and detected using specific antibodies.

nerg protein, inhibits megachannel opening, whereas Bax, a death agonist protein, facilitates the opening of megachannels (39). The increase in the content of agonists and the concomitant decrease in the content of antagonists stimulate the release of cytochrome c from the mitochondria, with the consequent activation of caspase activities.

Western blotting analysis, which is shown in Fig. 8, revealed that treatment of Y79 cells with 2 mM sodium butyrate for 24 h decreased the level of Bcl-2 and concomitantly increased the level of Bax. Moreover, a decrease in the mitochondrial level of cytochrome c was observed, whereas its cytosolic level simultaneously increased. However, treatment with 0.5 mM sodium butyrate induced only modest changes in the levels of Bcl-2, Bax, and cytochrome c. As shown in Fig. 8, treatment for 24 h with MG132 (0.2 or 0.5 μM) did not modify the level of Bcl-2 but increased the level of Bax. Moreover, MG132 also seemed to favor the release of cytochrome c from the mitochondria, because the mitochondrial level of cytochrome c appeared diminished after 24 h of treatment, and the cytosolic level increased. The addition of 0.2 or 0.5 μM MG132 to 0.5 and 2 mM sodium butyrate, respectively, did not modify the lowering effect of butyrate on the Bcl-2 level, whereas it enhanced the increasing effect on Bax level and the release of cytochrome c from the mitochondria (Fig. 8).

The Effects of a Combination of Sodium Butyrate and MG132 on Apoptosis and Caspase Activity. As reported above, the effects induced by both sodium butyrate and MG132 on apoptosis were dose dependent. At a dose of 0.5 mM, sodium butyrate had no effect on apoptosis, whereas 0.2 μM MG132 caused only a modest effect (Fig. 10A). However, when 0.5 mM sodium butyrate and 0.2 μM MG132 were added together, about 33% of cells showed clear morphological signs of death after 24 h of incubation, after which the number of apoptotic cells increased with time, reaching about 65% after 48 h. A similar synergistic effect was also observed with the activation of caspase-3. In fact, when 0.5 mM butyrate and 0.2 μM MG132 were used separately, only slight increases in the level of caspase-3 activity were found, whereas treatment of the cells with a combination of the two substances induced remarkable increases in the activity of caspase-3. This activity was 5 times higher than that of the control at 24 h of treatment and about 8 times higher than that of the control at 48 h of treatment (Fig. 10B).

These results were confirmed by flow cytometric analysis, showing that when suboptimal concentrations of sodium butyrate (0.5 mM) or MG132 (0.2 μM) were administered simultaneously to Y79 cells, the subdiploid population corresponded to about 30% after 24 h of
treatment, whereas this percentage was less than 8% in cells treated with 0.2 \mu M MG132 alone and about 3% (identical to control) in cells treated with 0.5 mM sodium butyrate alone (Fig. 11).

**DISCUSSION**

**Sodium Butyrate Induces Apoptosis in Y79 Cells.** We have previously demonstrated that a number of DNA-damaging compounds stimulated apoptosis in Y79 cells. The list includes cisplatin, carboplatin (19), and inhibitors of topoisomerase I (camptothecin and its analogues, irinotecan and topotecan) or topoisomerase II (etoposide and amsacrine; Ref. 18). With these compounds, apoptosis, which was anticipated by an enhancement in the p53 level, appeared after a lag period of 8 h of incubation and reached a maximum at 24 h.

This study demonstrates that sodium butyrate is a good inducer of apoptosis in Y79 cells, although its effect takes place more slowly than with DNA-damaging drugs and after a longer latent period. Butyrate-induced apoptosis was preceded by the activation of caspase-3, which was clearly observed at 24 h of treatment by direct estimation of its activity and by Western blotting analysis, showing the conversion of procaspase-3 to the active form of the enzyme. Activation of caspase-3 was accompanied by degradation by PARP with the production of the \( M_r 85,000 \) fragment. All these events preceded the appearance of the morphological signs of apoptosis, which were observed in a large percentage of cells only after 48 h treatment.

To explain the effect of sodium butyrate on the activation of caspase-3, we tested whether butyrate is capable of inducing changes in the level of cytochrome \( c \) in both the mitochondria and cytosol. The results authorize us to sustain that after 24 h of treatment cytochrome \( c \) was released from the mitochondria under the influence of sodium butyrate. Such an event could be responsible, as suggested recently, for the production of the apoptosome, with the consequent activation of caspase-9, which, in turn, activates caspase-3 (38). Our results tend to support the hypothesis that the release of cytochrome \( c \) is induced in butyrate-treated Y79 cells by the decrease in the level of the antiapoptotic factor Bcl-2 and the concomitant enhancement in the level of the proapoptotic factor Bax. Our finding that these modifications were not counteracted by \( z \)-VAD-fmk, an inhibitor of caspase activities, suggests that these effects preceded the activation of caspases. It is known that in treated cells, sodium butyrate induces histone hyperacetylation, favoring a relaxed state of chromatin structure with increased availability to transcription factors (7). In Y79 cells, such a mechanism could be responsible for the changes induced by sodium butyrate in the expression of the Bcl-2 and Bax genes. A similar conclusion was suggested by Litvak et al. (40), who demonstrated down-regulation of Bcl-2 and Bcl-X \(_L\) induced by sodium butyrate in Caco-2 cells.

The observation that in Y79 cells, butyrate also provoked the degradation of lamin B, a substrate of caspase-6 (29), suggested that other caspases besides caspase-3 could be involved in butyrate-induced apoptosis. This was confirmed by the finding that when Y79 cells were treated with sodium butyrate plus Ac-DEVD, a selective inhibitor of caspase-3, apoptosis was reduced but not suppressed, whereas when \( z \)-VAD-fmk, a general inhibitor of caspases, was used, all of the morphological signs of apoptosis were suppressed.

The decrease in the number of Y79 cells observed when the cells were treated with butyrate must be considered as a consequence not only of cell death by apoptosis but also of the proliferative arrest induced by butyrate itself. Such a conclusion has been suggested by the finding that \( z \)-VAD-fmk suppressed butyrate-induced apoptosis but did not completely abolish the effect on cell number, which remained lower than the control when the samples were simultaneously treated with sodium butyrate and \( z \)-VAD-fmk. The proliferative arrest induced by butyrate occurs in the G2-M phase of the cell cycle, as demonstrated by flow cytometric analysis. Our finding agrees with the observations of other authors (41, 42) that butyrate favors the arrest of the cell cycle in G2-M phase when pRB is absent in the cells, although it is capable of inducing cell cycle arrest in G1-S phase when functional pRB is present. Our results also show that in Y79 cells, butyrate increased the expression of p21/WAF1, an effect that was independent of p53 and was most likely responsible for the proliferative arrest of the cell cycle. Moreover, our observation that sodium butyrate already lowered the number of cells on the first day of treatment strongly suggests that the effect of butyrate on the cell cycle preceded the induction of apoptosis.

**Activation of Proteasome by Sodium Butyrate.** 26S proteasome has a central role in the cleavage of cellular proteins and is responsible in particular for the cleavage of many regulatory short-lived proteins, which include oncogenic products (c-fos and N-myc; Ref. 33), tumor suppressor factors (p53 and p27; Ref. 31, 43), cyclins (44), and members of the NF-kB family such as p105, the precursor for p50 NF-κB (45). 26S proteasome is a complex composed of two 19S components that recognize ubiquitinated proteins and a large central structure, the 20S proteasome (46). The latter contains multiple proteolytic sites that have chymotrypsin-like, trypsin-like, or caspase-like specificities and are capable of assuring the complete cleavage of proteins with the production of small peptides. 26S proteasome can be stimulated by many exogenous compounds such as cardiolipin, gangliosides, or fatty acids, which modify the kinetic parameters of the peptidase activities (48, 49). On the other hand,
many compounds are capable of inhibiting 26S proteasome, among which peptide aldehydes such as MG132 and MG115 are particularly effective, readily entering the cells and inhibiting the chymotrypsin-like activity (50).

Treatment of Y79 cells with sodium butyrate caused a remarkable decrease in the levels of some short-lived proteins such as p53, N-myc, and IкBα, whereas the nuclear level of NFκB was enhanced. The finding that all these effects were suppressed by the addition of MG132 suggested that butyrate stimulated the activity of 26S proteasome in Y79 cells. This fact has been demonstrated by us for the first time by means of the direct estimation of the proteolytic activity of 26S complex. On the other hand, MG132 did not modify the effect exerted by butyrate on the level of Bcl-2, thus suggesting that the decrease in the Bcl-2 level cannot be considered a consequence of proteasome activation.

Evidence in the literature suggests that the decrease in p53, N-Myc, and IкBα levels and the enhancement in the nuclear level of NFκB could exert an antiapoptotic influence. To this end, it has been reported that mouse embryo fibroblasts derived from p53 knockout mice are refractory to Myc-induced apoptosis (51), and that even in a p53-independent mechanism, the loss of p53 delays the appearance of c-Myc-induced apoptosis (52). Moreover, nuclear NFκB, which behaves as a transcriptional factor, prevents the cells from apoptosis induced by tumor necrosis factor and other factors (53), favoring the expression of some genes (TRAF-1 and -2 and cIAP-1 and -2) and the consequent suppression of caspase-8 activation (54). Accordingly, it has also been demonstrated that when the nuclear translocation of NFκB was prevented by lactacystin (an inhibitor of proteasome activity), lymphocytes of human chronic lymphocytic leukemia became sensitive to apoptosis by tumor necrosis factor (55).

MG132 Induces Apoptosis in Y79 Cells. It has been ascertained that inhibitors of 26S proteasome are capable of triggering apoptosis in rapidly dividing cells (56). Such an effect has been correlated with the ability of proteasome inhibitors to increase the intracellular levels of many short-lived factors (31, 43). However, it has also been suggested that apoptosis induced by proteasome inhibitor can be a consequence of activation of c-Jun NH2-terminal kinase (57), an enzyme that is involved in the initiation of programmed cell death.

Results presented in this study clearly demonstrate for the first time that in Y79 cells, MG132, a powerful inhibitor of proteasome, induced apoptosis together with remarkable increases in the levels of some short-lived proteins such as p53, IкBα, and particularly N-myc. These factors, which are usually cleaved by proteasome activity, play stimulatory roles in apoptosis. In addition, our results demonstrated that MG132 increased the level of Bax protein, a factor that seems to be involved in p53-mediated response because it favors the release of cytochrome c from the mitochondria into the cytosol and the consequent activation of caspase-3. The increase in the level of IкBα could also have an apoptotic effect, preventing NFκB translation into the nucleus.

Moreover, this study presents evidence that MG132, like sodium butyrate, lowered the level of E2F. A role in apoptosis has been suggested for E2F in cells lacking pRb (14). It has been reported that the activation of E2F could result in apoptosis when placed in conflict with an arrest of the cell cycle caused by an apoptotic agent (58). However, at the moment, it is not known whether a decrease in E2F, as seen in Y79 cells, can counteract the effectiveness of apoptosis induced by sodium butyrate and MG132.

The Synergistic Effects of the Association between Sodium Butyrate and MG132. All of the results reported in this study support the conclusion that sodium butyrate lowers the levels of p53, N-myc, and IкBα (factors that play stimulatory roles in apoptosis) through the activation of 26S proteasome. Such an event could be responsible for the delay with which butyrate-induced apoptosis occurs in Y79 cells. The finding that MG132, an inhibitor of proteasome activity, also induced apoptosis in Y79 cells and concomitantly increased the levels of p53, N-myc, and IкBα suggested a possible synergistic interaction between sodium butyrate and MG132 in the induction of apoptosis. This postulate was confirmed by our results, which showed synergistic effects between suboptimal amounts of sodium butyrate and MG132 on both apoptosis and activation of caspase-3. These effects were probably a result of the inhibition of proteasome activity by MG132 with the suppression of the stimulatory action of butyrate. Consequently, the cells were prevented from decreasing their levels of p53, N-myc, and IкBα. The presence of adequate amounts of these stimulatory factors most likely enhanced cell susceptibility to the induction of apoptosis by sodium butyrate.

Moreover, when the cells were simultaneously exposed to MG132 and butyrate, the level of Bax protein increased remarkably. Bax protein is the product of a gene that represents a transcriptional target of p53. Thus, the increased level of p53 in cells exposed to combined treatment can potentiate the effects of butyrate on the expression of Bax protein. In addition, in these cells, Bcl-2 remained at the low level found in cells treated with butyrate alone. The increase in the Bax level and the concomitant decrease in the Bcl-2 level could be responsible for the enhanced release of cytochrome c from the mitochondria, with the consequent activation of caspase-3 and the induction of apoptosis.

The induction of apoptosis has been recognized as an effective tool in the therapeutic treatment of many forms of tumors, and apoptosis can be triggered by a number of chemotherapeutic agents. However, the efficacy of treatment with apoptotic drugs is limited by their toxicity and the appearance of cell resistance to them (59). Sodium butyrate, a natural product capable of inducing apoptosis in a number of tumor cells, exhibits a low degree of clinical toxicity (60). Therefore, the association of sodium butyrate with other compounds, which enhance its capacity to act on apoptosis, can represent a tool of particular importance in tumor therapy. In this regard, a synergistic effect has recently been reported by Medina et al. (4), who observed that cells primed with butyrate were rendered highly susceptible to apoptosis by staurosporine, an agent that causes mitochondrial release of cytochrome c. Our results regarding the synergistic apoptotic effects of sodium butyrate and proteasome inhibitor MG132 may open new and interesting perspectives in the therapeutic strategy for treatment of human retinoblastoma. It is of interest that the inhibitors of proteasome seem to behave as apoptotic agents only in rapidly dividing cells, whereas they protect quiescent cells from apoptosis triggered by many different compounds (56, 61). Due to this particular behavior, proteasome inhibitors may represent a new alternative in the treatment of some proliferative diseases. In some cases, proteasome inhibitors have toxic effects, but in the future, other compounds in this family with lower toxicity could be used efficaciously to inhibit the growth of cells. Among them, a new class of compounds showing a boronate group linked to the peptide sequence seems particularly promising (50). In addition, associations between a proteasome inhibitor and other effective agents of apoptosis could induce a clear effect at lower concentrations of the compounds, as shown in this study, thus reducing the toxicity of the therapeutic treatment.

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The Apoptotic Effects and Synergistic Interaction of Sodium Butyrate and MG132 in Human Retinoblastoma Y79 Cells

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