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BUTYRIC ACID AND ITS MONOSACCHARIDE ESTER INDUCE APOPTOSIS IN THE HL-60 CELL LINE

C. Calabresse[1,3], L. Venturini[1], G. Ronco[2], P. Villa[2], C. Chomienne[1] and D. Belpomme[3]

[1] *Laboratoire Université Paris 7 (Biologie Cellulaire Hématopoïétique) Hôpital Saint-Louis, Paris , France*

[2] *Université de Picardie, Amiens , France*

[3] *Centre de recherche de l'ARTAC, Hôpital Boucicaut, Paris , France*

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SUMMARY: Butyric acid is a potent cell growth inhibitor and differentiation inducer (1-4). However, the short plasma half-life of this fatty acid limits its potential therapeutic use (5,6). The recent synthesis of several monosaccharide esters of butyric sodium salt (BuONa) with a prolonged plasma half-life and similar biological properties as the sodium salt opens new perspectives (7-12). We report here that the human myeloid HL-60 cell line can be induced to apoptosis when cultured with one of these esters, monoacetone glucose 3-butyrate (MAG=3but) or BuONa. Cytospin slide preparations and flow cytometric studies showed that HL-60 cells treated with 1 mM MAG=3but or BuONa exhibited a reduction in cell volume and condensation of nuclear structure characteristic of apoptosis, associated with monocytic differentiation. Time course studies demonstrated that DNA fragmentation, as determined by agarose gel electrophoresis, was detected 4 hours after incubation with the drugs, while morphologic signs appeared at day 3. Apoptotic cells increased with culture time and reached a maximum at day 6 of 20 ± 5 % with BuONa, 25 ± 5% with MAG=3but, and only 5 ± 2% in controls. These findings suggest that these drugs may exert their actions, at least in part, through induction of apoptosis.

Abbreviations: BuONa, butyric sodium salt; MAG=3but, monoacetone glucose 3-butyrate; ATRA, all-trans retinoic acid; NBT, nitroblue tetrazolium.

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Previous studies have shown the pharmaco-therapeutic properties of butyric esters derived from natural polyhydroxylated compounds (7-12). These stable butyrate derivatives could improve the rapid turnover and the therapeutic action of butyric salts. Indeed, the lack of clinical efficacy of the latter has been attributed mainly to rapid metabolism (13). One of the butyric esters has been chosen for its high water-solubility and absence of bad odor originally produced by butyric salts (7), its limited toxicity in rodents (8), its antitumoral action in vitro and in vivo (9-11) and its particularly interesting pharmacokinetics (12).

Although some studies have shown that inhibition of DNA synthesis by BuONa is associated with a hyperacetylation of histones H3 and H4 due to an n-butyrate induced decrease in activity of some histone desacetylases (14,15), the mecanism of butyric acid is still unknown.

Recently, a variety of chemotherapeutic agents such as taxol or all-trans retinoic acid (ATRA) have been demonstrated to induce leukemic cell death by apoptosis (16,17).

Apoptosis play an important role in embryogenesis (18), carcinogenesis (19), hormone-dependent atrophy of tissues and tumors (20), immune system regulation (21) and cell survival promoted by hematopoietic colony-stimulating factors (22).

Cells undergoing apoptosis are characterized by several morphological and biochemical events, such as cellular shrinkage, chromatin condensation, DNA fragmentation and formation of apoptotic bodies (23,24).

Apoptosis can be induced by various physical and chemical stimuli in the human myeloid cell line HL-60 (25-28). In contrast to what has been observed in mature thymocytes (29,30), no requirement for RNA or protein synthesis seems necessary for the apoptotic process in HL-60 cells (31,32).

In the present paper, we report that sodium butyrate as well as its monosaccharide ester, MAG=3but, can induce accelerated apoptosis in the human myeloid HL-60 cell line.

Cells

Cells were seeded at an initial concentration of $2x10^5$/ml in RPMI 1640 medium containing 15% (v/v) fetal calf serum (FCS), 100 IU/ml penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine. Cells were maintained at 37°C in an atmosphere of 95% air, 5% CO_2. BuONa (Sigma chemical Co, Saint Louis, MO) or MAG=3but (ARTAC, France) were added at the beginning of the culture. Stock solutions were stored at -20°C.

Cell viability

Cells were counted on a Malassez hemocytometer and were assessed for viability by trypan blue exclusion test.

Differentiation assay and histochemical staining

To quantify differentiation, the nitroblue tetrazolium (NBT) reduction test was performed using standard methodology with phorbol myristate acetate (PMA), and the percentage of cells containing intracellular black formazan deposits determined as previously described (33). Cytospin slide preparations of untreated and BuONa- or Mag=3but-treated cells were stained with May-Grünwald-Giemsa stain for evaluation of nuclear morphology and cellular differentiation. Counts were performed under light microscopy on a minimum of 200 cells.

DNA extraction

At the end of the culture, cells ($10x10^6$/ml) were washed twice in PBS at 4°C. The pellets were lysed with 2 ml of lysis buffer (50 mM Tris-Hcl pH 8/ 20 mM EDTA pH 8/ 2% SDS), incubated at 37°C for 18 hours and chilled on ice for 10 minutes. To precipitate proteins, 0.8 ml of a satured NaCl solution was added and tubes were left on ice for 5 minutes. Precipitates were centrifuged at 3000 rpm for 1 hour to separate intact from fragmented chromatin. RNase was then added to supernatants at a final concentration of 20 µg/ml and incubation at 37°C was continued for 15 minutes. DNA was then precipitated by adding two volumes of 100% ethanol.

DNA electrophoresis

Prior to electrophoresis, loading buffer (containing 0,25% bromophenol blue, 0,25% xylene cyanol, 15% ficoll) was added to each sample (40 µg DNA) at a 1:5 (v/v) ratio. DNA was electrophoresed in a 1.25% agarose gel overnight. Electrophoresis was carried out in 2 mM EDTA pH 8/ 89 mM Tris/ 89 mM boric acid (TBE). A Hind III- digest of λ-DNA and Hae III- digest of Φ-DNA was used as molecular size standard of 23.13; 9.4; 6.6; 4.4; 2.3; 2; 1.3; 1; 0.87; 0.6; 0.56; 0.31; 0.28; 0.27; 0.23; 0;19; 0.12; 0.11; 0.07 Kbp. After the completion of electrophoresis, DNA in the gel was visualized by soaking the gel for 1 hour in TBE buffer containing 1 µg/ml ethidium bromide and destained briefly in distilled water. The stained gel was viewed by transillumination with UV light (302 nm) and photographed.

RESULTS

Growth of HL-60 cells was inhibited by the presence of 1 mM MAG=3but or BuONa concentration (Fig. 1). This inhibition appeared on the second day and reached the highest level on the 6th day, 70±2% with MAG=3but and 40±5% with BuONa, compared to controls. Culturing cells in the presence of lower MAG=3but or BuONa concentration produced no effect on HL-60 cell growth while incubation with higher concentration resulted in a rapid loss of cell viability (data not shown).

HL-60 cells grown under the influence of 1 mM BuONa or 1 mM MAG=3but exhibited morphological changes characteristic of more mature

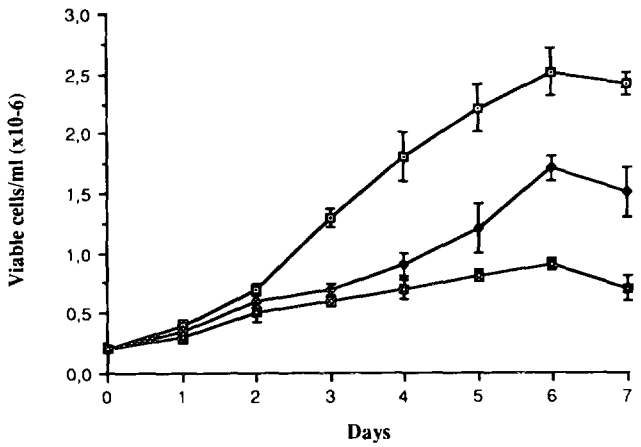


Fig. 1. Time course studies of HL-60 viability. Cells were incubated in medium alone (-□-), with 1 mM BuONa (-●-), 1 mM MAG=3but (-■-). Results are expressed as the mean (± s.e.m) of three experiments.

cells in the monocytic pathway (Fig. 2). Differentiation, assessed by the NBT test, showed an increased percentage of differentiated cells from day 3 to day 6 where it reached 50±5% for BuONa and 60±7% for MAG=3but (Fig. 3).

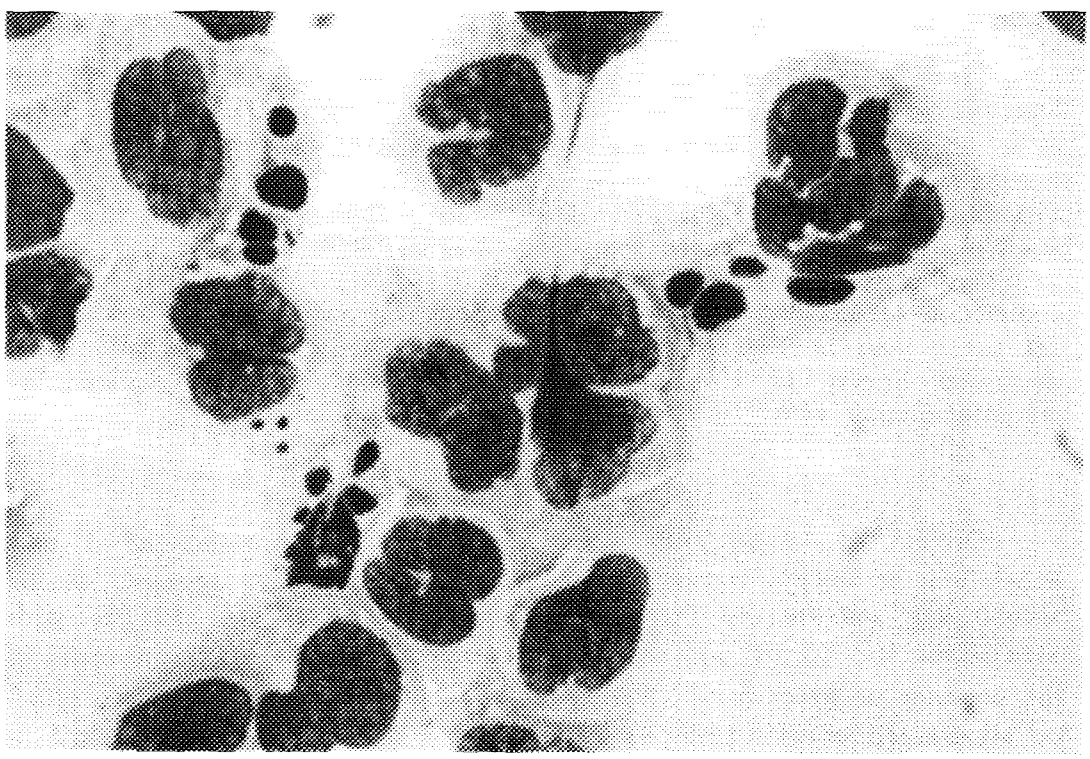


Fig. 2. Morphological appearance of HL-60 cells treated for 6 days with 1 mM MAG=3but. Note the morphology of apoptotic cells with their highly condensed chromatin and apoptotic bodies.

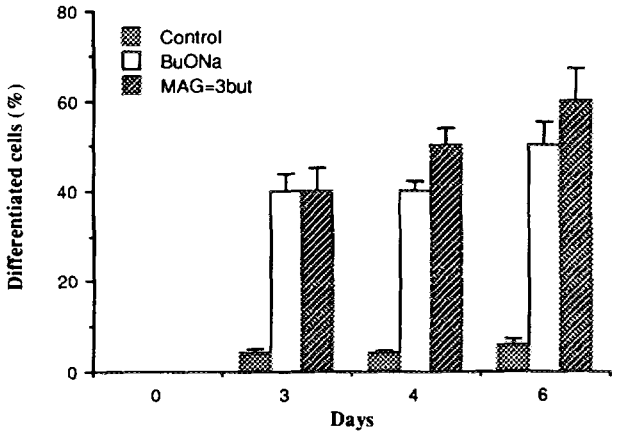


Fig. 3. Time course of appearance of differentiated cells, assessed by NBT assay in HL-60 cultures in the presence or absence of 1 mM BuONa or 1 mM MAG=3but. The results are expressed as the mean (± s.e.m) of three experiments.

At the same time, in culture, a feature of cells with highly condensed chromatin and fragmented nuclei appeared (Fig. 2). These features were characteristic of apoptotic cells as described extensively (24). At a specific concentration of 1 mM MAG=3but or BuONa, by day 6, 20±5% (for BuONa) or 25±5% (for MAG=3but) of HL-60 cells presented signs of apoptosis, as compared to only 5±2% for controls (Fig. 4). It is interesting to note that with lower or higher concentrations, HL-60 cells did not exhibit significant levels of apoptotic cells compared to controls, even though at higher concentrations (3mM) an increased cytotoxicity was observed (data not shown).

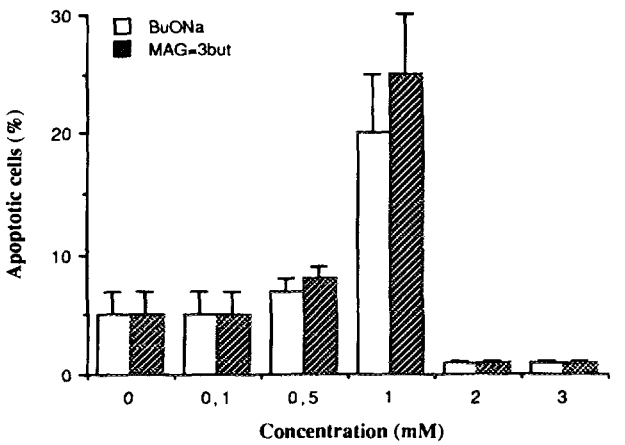
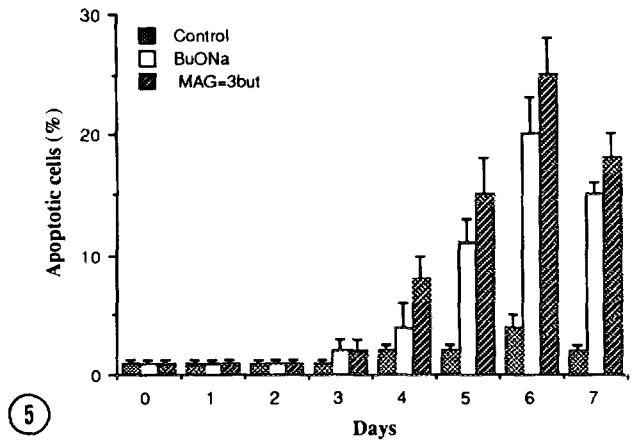


Fig. 4. Percentage of HL-60 cells with morphological appearance of apoptosis after culture for 6 days in the presence of BuONa or MAG=3but at concentrations from 0.1 to 3 mM. The results are shown as mean (± s.e.m) of three experiments.



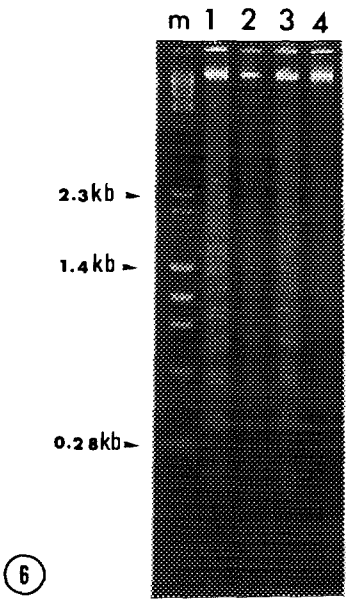


Fig. 5. Percentage of apoptotic HL-60 cells, assessed morphologically at time points up to 7 days in the presence or absence of 1 mM BuONa or 1 mM MAG=3but. The values represent the average of three separate determinations (± s.e.m).

Fig. 6. Agarose gel electrophoresis demonstrating DNA fragmentation in HL-60 cells after 4 h of culture. *m*: DNA size markers, *Track 1*: cells treated with 1 mM MAG=3but, *Track 2*: cells treated with 1 mM BuONa, *Track 3*: cells treated with 10^{-6} M ATRA, *Track 4*: control cells.

Furthermore, HL-60 cells, after incubation with 1 mM BuONa or MAG=3but, presented increased morphological characteristic of apoptosis in a time-dependent manner. The percentage of apoptotic cells was detectable from day 3 and reached a maximum at day 6 (Fig. 5).

Because the process of apoptosis is associated with DNA degradation in many models, agarose gel electrophoresis of DNA extracted from treated cells was performed. After only 4 hours of incubation with the drugs, DNA cleavage bands with a characteristic pattern of the internucleosomal ladder were detected (Fig. 6), thus DNA degradation was preceding morphological signs of apoptosis.

DISCUSSION

The results presented herein show that MAG=3but inhibits HL-60 cell growth more significantly than BuONa. At the appropiate concentration (1 mM), both induce pronouncedly apoptosis in HL-60 cells (Fig. 4), whereas at higher concentrations (3 mM), they suppress DNA fragmentation even though they display an increased cytotoxicity (data not shown). This suggests

that other mechanisms may be involved at higher drug concentrations and that endonucleases or factors essential to apoptosis are activated only at lower concentrations. Apoptosis, assessed both by morphology and evidence of DNA fragmentation, was shown to be time dependent. Time course studies demonstrated that DNA fragmentation was detected 4 hours after incubation with the drugs (Fig. 6), while morphological signs appears at day 3 (Fig. 5).

We also showed that MAG=3but-induced apoptosis in HL-60 cells was associated with differentiation through the monocytic pathway, at the same concentration (Fig. 3).

Up to now, the exact mechanism of action of butyrate is unknown. It has been demonstrated that, in a variety of mammalian cells, butyrate induces an hyperacetylation of histones H3 and H4 due to an inhibition of desacetylase activity (14,15). Furthermore, it has been proposed that acetylation of histones releases constraints upon the DNA template and that regions of DNA in association with hyperacetylated histones are preferentially solubilized upon digestion with DNAse I (34).

Recently, the endogenous deoxyribonuclease involved in nuclear DNA degradation during apoptosis has been characterized as functionally and antigenically indistinguishable from DNAse I (35). This provides a possible mechanism for the observation that the monosaccharide ester of BuONa, MAG=3but, induces apoptosis in the HL-60 cell line.

In conclusion, these findings could be the first step of understanding the mechanism of action of butyrate.

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