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Rapid alteration of c-*myc* and c-*jun* expression in leukemic cells induced to differentiate by a butyric acid prodrug

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The novel prodrug of butyric acid (BA), pivaloyloxymethyl butyrate, has been shown, in vitro, to induce differentiation and inhibit leukemic cell proliferation. The prodrug affects the cells in vitro at lower concentration and at least 100 times faster than does (BA). We have compared the ability of BA with that of its prodrug AN-9 to modulate the expression of the early regulating genes, c-*myc* and c-*jun*, in HL-60 cells. Exposure of HL-60 cells to the prodrug resulted in a decrease of c-*myc* and an increase of c-*jun* expression. The prodrug elicited this effect at lower concentrations and at least 100 times faster than BA. Since changes in the expression of c-*myc* and c-*jun* occur minutes after exposure of the cells to the prodrug, these genes are likely to play a major role in the early stages of the differentiation pathway.

c-*myc*; c-*jun*; Butyric acid; Butyric acid prodrug; Proliferation; Differentiation

1. INTRODUCTION

Leukemias are characterized by a block in the normal pattern of differentiation, leading to the accumulation of immature cells. Aberrant expression of oncogenes may play a causative role in the transformed state of the cells [1]. Deregulated c-*myc* expression plays a pivotal role in oncogenesis in a variety of experimental and naturally occurring tumors [2]. The promyelocytic HL-60 cells have an elevated level of c-*myc* expression, attributed to gene amplification at the DNA level and over expression at the RNA level [3,4]. Both c-*myc* and c-*jun* are early regulatory transcription factors with specific DNA binding motifs; c-*myc* has a helix-loop-helix motif and c-*jun* contains a leucine zipper dimerization motif. Homodimers of c-*jun* or heterodimers of c-*jun*/c-*fos* form the AP-1 protein that can recognize and bind to specific sequences of DNA nucleotides [1]. The expression of these genes is linked to proliferation of some cells and to the differentiation of others. They are among the first genes to be expressed when certain cells are stimulated to either proliferate or differentiate [5,6].

Butyric acid (BA) was shown to modulate the expression of oncogenes and suppresser genes in several cell types [7,8]. It is a potent differentiating and anti-proliferating agent, in a wide spectrum of neoplastic cells in vitro [9]; however, in vivo it lacks biological activity, due to rapid metabolism [10]. The novel derivative of BA, pivaloyloxymethyl butyrate (AN-9), has been shown, in vitro, to induce cytodifferentiation and inhibit leukemic cell proliferation. AN-9 affects the cells in vitro at about a 10-fold lower concentration and at least 100-fold faster than does BA [11,12]. While BA has no in vivo anti-cancer activity, AN-9 was shown to be an effective anti-cancer agent in murine cancer models.

In this report we have compared the effect of BA and its derivative AN-9, on the expression of the early regulatory genes, c-*myc* and c-*jun*, in HL-60 cells.

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2. MATERIALS AND METHODS

2.1. *Cell culture*

Human promyelocytic cell line HL-60 was grown as described [11]. Mycoplasma free cells were incubated at 37°C in a humidified CO_2 incubator. Viability was determined by Trypan blue exclusion.

2.2. *Cell proliferation*

Cell proliferation was measured by incorporation of [^{3}H]thymidine (2 Ci/mmol, Amersham, Little Chalfont, UK) into cells. Cells were seeded at a density of 5×10^4 cells/well in 96-well plates (Cell-Cult). During the last 14 h of incubation, the cells were pulsed with [^{3}H]thymidine, 1 μCi/well, and harvested (Microtiter Dynatech Cell Harvester) using glass micro fiber filters (Tamar, Israel).

2.3. *Cell differentiation*

Cell differentiation was evaluated by Nitroblue tetrazolium (NBT) reduction activity [13]. Cell culture containing 0.1% NBT was stimulated with 400 nM 12-*O*-tetradecanoyl-phorbol-13-acetate (PMA). The cells were incubated for 30 min at 37°C and examined microscopically by scoring at least 200 cells.

2.4. *RNA analysis*

All reagents used were of analytical or molecular biology grade. Total cellular RNA was isolated by acid guanidinium thiocyanate

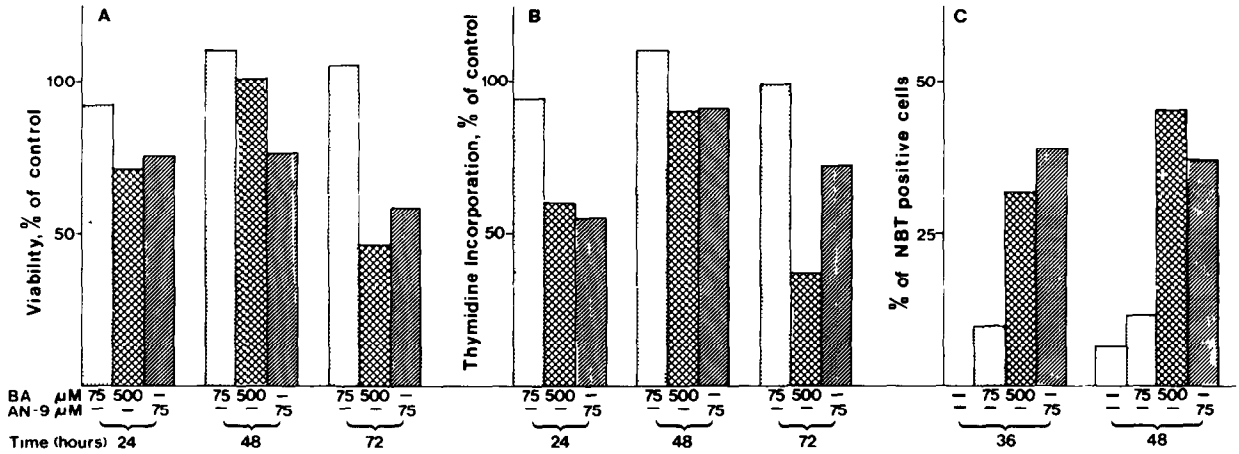

Fig. 1. The effect of BA and AN-9 at low (75 μM) and high (500 μM) concentrations on viability, proliferation and differentiation of HL-60 cells. The % of viable cells (A), thymidine incorporation (B) and % of NBT positive cells (C) were determined after exposure of the cells for the indicated time intervals. 100% thymidine incorporation (the incorporation value in untreated cells) was 35,000, 89,000 and 105,000 cpm at 24, 48 and 72 h, respectively.

phenol/chloroform extraction, according to Chomczynski and Sacchi [14]. RNA 20 μg/lane were size fractionated on agarose-formaldehyde gel (agarose from BRL, formaldehyde from Sigma) and transferred to Nytran filter (Schleicher & Schuell) [15]. The blots were hybridized to the following multiprime ^{32}P-labeled DNA probes: (1) the 1.4-kb *Cla*I-*Eco*RI fragment containing the third exon of human c-*myc* 3Rc [16]; (2) the 1.1-kb *Pst*I-*Eco*RI fragment of human c-*jun* [17]. An oligonucleotide probe of human 28S ribosomal RNA (rRNA) labeled at the 5'-end (Oncogene Science Cat #ON135) was used throughout these studies, to normalize variations in the amount of RNA in individual samples. Appropriate exposures of the autoradiographs were scanned using a Zenith Soft Laser scanning densitometer (model SLR-2D/1D). The level of RNA was quantified by determination of the area under each peak with a planimeter. The obtained values were normalized to the 28S rRNA level and estimated as a percent of the untreated cells. This value was termed 'relative integrated density' or RID.

3. RESULTS

3.1. *Effect of BA and its prodrug AN-9 on HL-60 proliferation and differentiation – comparative studies*

HL-60 cells were exposed to 75 μM (low) and 500 μM (high) concentrations of BA and AN-9, for various time intervals. The effect of this treatment on cell viability, proliferation and differentiation was determined (Fig. 1). At 75 μM, AN-9 moderately affected cell viability (60–80%), inhibited cell proliferation and induced cell differentiation, while BA at the same concentration did not exhibit any of the above effects. Cell differentiation was determined by the % of NBT positive cells, as described in section 2. The values obtained were: (a) after 24 h of exposure to the low AN-9 and the high BA concentrations the cells exhibited 19% and 13% respectively; (b) after 36 h the effect of low AN-9 concentration was maximal, 39% and the effect of the high BA concentration was 32%; (c) only after 48 h the BA effect was maximal, 46%, and the AN-9 effect started to decline slightly to 37% (Fig. 1B). The effectiveness of BA at 500 μM and AN-9 at 75 μM was similar, whereas AN-9 at 500 μM completely inhibited cell proliferation. At 500 μM, after prolonged exposure (24 h), AN-9 was toxic to the cells (data not shown). However a short (30–60 min) exposure of the cells to this concentration, resulted in a maximal differentiation induction [11]. Under these treatment conditions the effect of BA and AN-9 on the expression of c-*myc* and c-*jun* was examined.

3.2. *Modulation of* c-myc *and* c-jun *expression by BA*

HL-60 cells were exposed to 75 μM and 500 μM BA for various time intervals. The effect of these treatments on the expression of c-*myc* and c-*jun* mRNA, was followed by Northern analysis (Figs. 2 and 3). At brief cell exposure (0.5, 5 and 12 h) to the low concentration of BA (75 μM), no effect was seen on the expression of

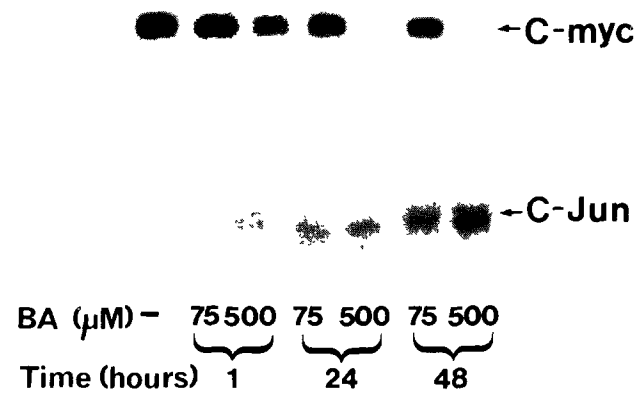

Fig. 2 Modulation of c-*myc* and c-*jun* expression, in HL-60 cells, by BA. Cells were treated with the indicated concentrations of BA for the indicated time periods. Total cellular RNA was isolated and analyzed for c-*myc* and c-*jun* expression as described in section 2.

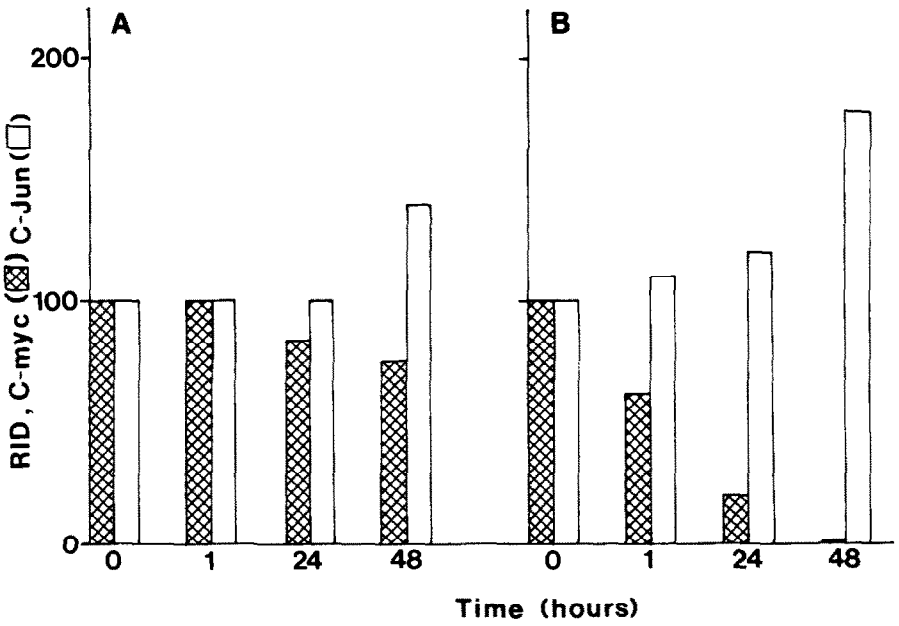

Fig. 3. Quantitative analysis of c-*myc* and c-*jun* expression. Northern blots were probed separately with: c-*myc*, c-*jun* and 28S rRNA. RID (relative integrated density) values were determined as described in section 2. A: 75 μM BA; B: 500 μM BA.

these genes (data not shown). A marginal effect (Figs. 2 and 3) at this low BA concentration was observed only after 24 and 48 h. At high BA concentration, the expression of c-*myc* was reduced to 63% of control at 1 h and disappeared at 48 h.

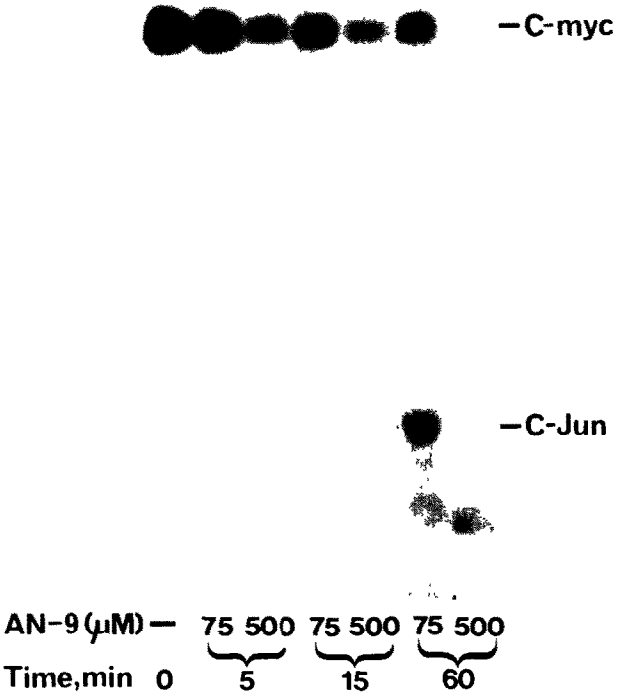

Fig. 4. Modulation of c-*myc* and c-*jun* expression in HL-60 cells by AN-9. The cells were treated with AN-9 at the indicated concentrations and time intervals. Total cellular RNA was isolated and analyzed for c-*myc* and c-*jun* expression.

The expression of c-*jun* was enhanced significantly only after 48 h of exposure, to low and high BA concentrations. Throughout the duration of the experiment, the steady state level of c-*myc* and c-*jun* mRNA, in untreated cells, did not vary significantly (data not shown).

3.3. *Modulation of* c-myc *and* c-jun *expression by AN-9*

Exposure of HL-60 cells to the low AN-9 concentration resulted in a decrease in c-*myc* and an enhancement in c-*jun* expression. AN-9 elicited these effects faster and at lower concentrations than BA. The following changes in the steady state level of the c-*myc* transcript in the cells, were observed: (a) after 15, 30 and 60 min exposure to 75 μM AN-9 respective decreases of 23%, 57% and 72%, followed by complete disappearance at 5 h, which continued at least up to 24 h; and (b) reappearance after 48 h (Figs. 4, 5 and 6). At 500 μM after 5 min a 72% reduction of c-*myc* transcript occurred and at 60 min the gene transcript was undetectable.

AN-9 at 75 μM enhanced the expression of c-*jun* transcript after 15 min of exposure and a maximum expression was achieved at 48 h. The steady-st[illegible] level of c-*jun* transcript expression which was low in untreated cells, was somewhat reduced after 15 and 30 min following the exposure of the cells to 500 μM AN-9.

4. DISCUSSION

BA has been shown to be an effective inducer of cytodifferentiation in a wide spectrum of neoplastic cells in vitro; however, in vivo, it exhibited low potency due to rapid metabolism [9,10]. Therefore, it is practically

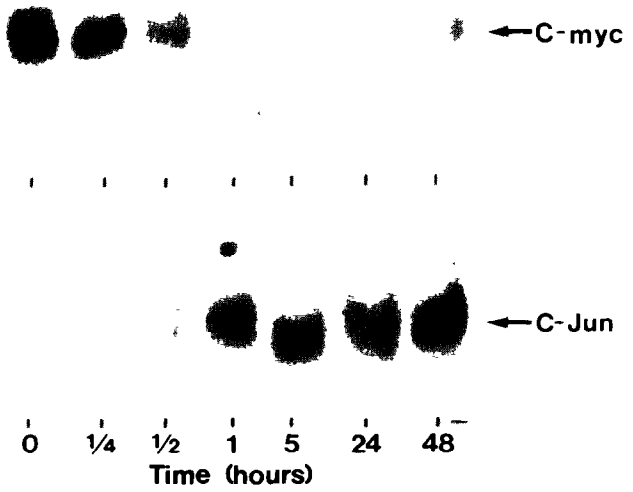

Fig. 5 The effect of low concentration of AN-9 on c-*myc* and c-*jun* expression in HL-60 cells. Cells were treated with 75 μM AN-9 and total cellular RNA was isolated at the indicated periods of time and analyzed for c-*myc* and c-*jun* expression.

impossible to achieve an effective BA concentration in vivo. Previously we have demonstrated that AN-9 induces cell differentiation faster and at lower concentration than BA and most importantly possesses an in vivo anti-cancer activity [11,12]. In this study we have compared the ability of BA with that of AN-9 to modulate the expression of genes which are known to have a pivotal role in the differentiation pathway.

Differentiation of leukemic cell lines was shown to be associated with a decrease in c-*myc* expression which is necessary and possibly sufficient for induction of cell differentiation [18]. The c-*myc* and c-*jun* proto-oncogene products, which associate to form heterodimeric transcription factors, are among the first genes to be expressed when cells are stimulated to proliferate or differentiate [19].

AN-9 induces cell differentiation faster than BA (Fig. 1C). This is in agreement with our previous results which have shown that brief cell exposure (less than 1 h) to AN-9 resulted in cell differentiation [11], while only prolonged exposure to BA (48 h), brought about a similar effect.

At optimal concentration, required for the differentiation induction of HL-60 cells, AN-9 (75 μM) and BA (500 μM) bring about a decrease in c-*myc* and an increase in c-*jun* steady-state level of expression. After exposure of the cells to BA and AN-9, a half-maximal change in the level of c-*myc* expression occurred after 15 h and 0.5 h respectively. Thus AN-9 at a lower concentration acted 30-fold faster than BA. A half-maximal change in the expression of c-*jun* transcript was achieved after 35 h of cell exposure to BA but only 0.5 h exposure to AN-9. AN-9 enhanced the expression of c-*jun* 70-fold faster than BA. These observations suggest the involvement of c-*myc* and c-*jun* in the differentiation induction mechanism of BA and its prodrug. Further-

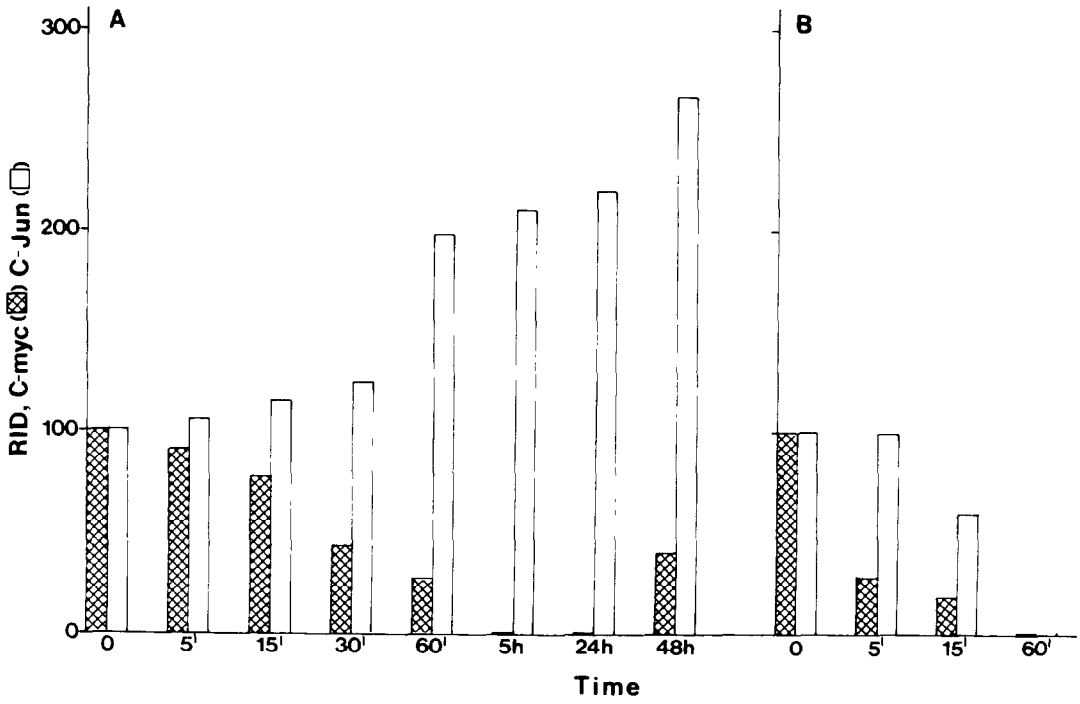

Fig. 6. Quantitative analysis of c-*myc* and c-*jun* expression. Northern blots probed separately with c-*myc*, c-*jun* and 28S rRNA. Quantitative analysis of c-*myc* and c-*jun* mRNA levels was conducted by densitometric scanning as described in section 2. A: 75 μM AN-9; B: 500 μM AN-9.

more, AN-9 most likely affects the cells by the same mechanism that BA does, but is far more effective.

Reappearance of c-*myc* was observed after 48 h. Rapid suppression and subsequent re-expression of c-*myc* was observed previously and was suggested to be involved in determining the kinetics of cell commitment to terminal differentiation [20,21]. At high concentration of AN-9, a dramatic change in the expression of c-*myc* took place (72% inhibition compared to untreated cells) following a 5 min exposure of the cells to the agent and at 1 h c-*myc* expression was undetected.

Unlike the parallel reciprocal changes observed in c-*myc* and c-*jun* expression, at the optimal concentration required for differentiation induction, a high concentration of AN-9 does not enhance c-*jun* expression. The initially low expression level of c-*jun*, at a high concentration of AN-9 (500 μM), is further reduced and becomes undetectable after 1 h. The differences in the pattern of gene expression following treatment with low and high AN-9 concentrations, can be explained by the differences in the cells' fate. While the treatment with a low concentration of AN-9 leads to cell differentiation, with a high concentration of AN-9 it causes cell death. The mechanism by which AN-9 (at the high concentration) induces cell death, remains to be determined.

The kinetics of BA and AN-9 induced modulation of gene expression, showed that the changes exerted by AN-9 are faster than those exerted by BA. This is consistent with AN-9's rapid mode of action and further supports the notion that it acts as a true BA prodrug. The superior pharmacokinetics of AN-9 may stem from either a faster rate of intracellular penetration by the lipophilic AN-9 and/or a slower rate of metabolic degradation. The in vivo anti-cancer activity of AN-9 might be attributable to its pharmacokinetic properties.

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