Cancer Chemother Pharmacol (1998) 41: 252–255

SHORT COMMUNICATION

Reynald Gillet · Pierre Jeannesson · Houcine Sefraoui
Marie-Luce Arnould-Guérin · Serge Kirkiacharian
Jean-Claude Jardillier · François Pieri

Piperazine derivatives of butyric acid as differentiating agents in human leukemic cells

Received: 1 June 1997 / Accepted: 23 July 1997

Abstract Butyric acid is a potent antineoplastic agent with a well-documented differentiation activity on a wide variety of tumor cells. However, its clinical development is strongly limited by its very short metabolic half-life. In this study we report on the in vitro effects of new original piperazine derivatives of butyric acid on the induction of differentiation and the growth inhibition of human erythroleukemia K562 cells and myeloid leukemia HL60 cells. 1-(2-hydroxyethyl) 4-(1-oxobutyl)-piperazine (HEPB) and [1-(2-hydroxyethyl) 4-(1-oxobutyl)-piperazine] butyrate (HEPDB) were efficient in acting on the differentiation and proliferation of both cell lines, whereas 1-phenyl 4-(1-oxobutyl)-piperazine (PPB) and 1-(3,4-methylene dioxybenzyl) 4-(1-oxobutyl)-piperazine (POB) acted only on proliferation rates. Such derivatives did not induce significant toxicity in mice. These preliminary results should enable, by the development of new series of piperazine derivatives, a better understanding of the mechanisms of action of butyric acid and its analogues on the coupling of growth and differentiation of neoplastic cells.

Key words: Butyric acid · Differentiation · Leukemia · Piperazine derivatives · Proliferation

R. Gillet · P. Jeannesson (✉) · J. C. Jardillier
Laboratoire de Biochimie et Biologie Moléculaire, Faculté de Pharmacie, IFR 53, Centre des Biomolécules, 51 rue Cognacq-Jay, F-51096 Reims Cedex, France
Tel.: +33 (0) 3 26 05 35 67
Fax: +33 (0) 3 26 05 37 30

F. Pieri · H. Sefraoui · M. L. Arnould-Guérin
Laboratoire de Pharmacologie, Faculté de Pharmacie, Université de Picardie, 1 rue des Louvels, F-88037 Amiens Cedex 1, France

S. Kirkiacharian
Laboratoire de Chimie Thérapeutique, Faculté de Pharmacie, rue Jean-Baptiste Clément, F-92226 Châtenay-Malabry, France

Introduction

Butyric acid, a natural short-chain fatty acid, has been shown to induce a cytodifferentiation and/or apoptosis program in a wide variety of neoplastic cells, including solid tumor lines and leukemia cells [4, 14, 16, 22, 24, 29]. Moreover, it can also be used in the treatment of β-chain hemoglobinopathies (sickle-cell anemia and β-thalassemia) because of its specific induction of fetal hemoglobin synthesis [3, 17, 18]. These properties give to butyric acid a real significance in the clinical treatment of neoplastic diseases and hemoglobinopathies, but, unfortunately, in spite of its strong and promising in vitro effects, its clinical use is limited because of its rapid metabolism; its very short half-life does not permit the achievement of effective doses in vivo and limits its therapeutic development [11, 15]. The use of butyrate derivatives and, more particularly, prodrugs of butyric acid seems to be one of the most promising ways to overcome this limitation [6, 13, 20, 21, 23, 27, 28]. In the present study we aimed at synthesizing new original piperazine derivatives and evaluating in vitro their biological activities on human leukemic cells. Erythroid differentiation was assessed on human erythroleukemia K562 cells and granulocytic differentiation, on human myeloid leukemia HL60 cells. Growth inhibition was also evaluated for both cell lines. We report on the effects of two compounds, 1-(2-hydroxyethyl) 4-(1-oxobutyl)-piperazine (HEPB) and [1-(2-hydroxyethyl) 4-(1-oxobutyl)-piperazine] butyrate (HEPDB), which display their activity by acting on cell growth and on the differentiation rate of both cell lines, and on the effects of two other compounds, 1-phenyl 4-(1-oxobutyl)-piperazine (PPB) and 1-(3,4-methylene dioxybenzyl) 4-(1-oxobutyl)-piperazine (POB), which do so by acting only on cell growth. These preliminary results should permit a better understanding of the structure-function relationship of butyrate derivatives on the coupling of growth and differentiation of neoplastic cells.

Materials and methods

Cell lines

K562 and HL60 leukemia cells were grown in suspension culture in RPMI-1640 medium (Life Technologies, Cergy-Pontoise, France) supplemented with 10% and 15% heat-inactivated fetal calf serum (Life Technologies, Cergy-Pontoise, France), respectively, and with 2 m*M* L-glutamine. Cells were maintained at 37 °C in a humidified atmosphere containing 5% CO_2.

Drugs

Butyric acid was obtained from Sigma Chemical Co (L'Isle d'Abeau Chesnes, France). Derivatives (Fig. 1) were synthesized from 1-phenylpiperazine for PPB, 1-piperonylpiperazine for POB, and *N*-β-hydroxyethylpiperazine for HEPB and HEPDB. Amidification of the amine group of piperazine compounds by butyric acid chloride was performed in dichloromethane as a solvent. The reagents were commercially available. The infrared spectrum has been recorded on a Perkin-Elmer 983 spectrophotometer and the [^{1}H]nuclear magnetic resonance (NMR) spectrum in CDCl3, on a Bruker spectrophotometer at 250 MHz using tetramethylsilane as an internal standard; the chemical shifts are expressed in parts per million [19]: *PPB* – m.p. 198 °C; [^{1}H]NMR: 0.98 (t,3H), 1.66 (m,2H), 2.28 (t,2H), 3.00 (t,4H), 3.45 (t,4H), 6.73 (m,5H); IR: 1624 cm^{-1}. *POB* – m.p. 80 °C; [^{1}H]NMR: 0.98 (t,2H), 1.62 (m,2H) 2.27 (t,2H), 2.42 (t,4H), 3.42 (s,2H), 3.60 (t,4H), 5.89 (s,2H), 6.67 (d,2H), 6.82 (s,1H); IR: 1624 cm^{-1}. *HEPB* – [^{1}H]NMR: 0.86 (t,2H), 1.49 (m,2H), 2.28 (t,2H), 2.5 (m,8H), 3.5 (m,6H), 4.1 (s,H); IR: 1623 cm^{-1}. *HEPDB* – [^{1}H]NMR: 0.86 (t,6H), 1.7 (m,2H), 2.56 (t,2H), 2.57 (t,4H), 3.57 (s,4H), 3.79 (t,4H), 4.1 (t,4H).

The respective salts (chlorides) were prepared for biological assays; aqueous stock solutions (100 m*M*) were sterilized through 0.22-μm filters (Millipore) and kept at –20 °C. They were diluted in culture medium immediately before use.

Assays for growth and differentiation

Cells in the exponential growth phase were incubated at 5×10^4/ml and treated with each of the derivatives for 96 h. The cell viability and cell number were determined by the trypan blue dye-exclusion method. Growth inhibition was calculated from: $[(C_4–C_0)–(T_4–T_0)/(C_4–C_0)]\times100$, where C_0, C_4, T_0, and T_4 represent the number of cells counted per milliliter in untreated (*C*) and treated (*T*) cultures at days 0 and 4, respectively. Differentiation was assessed as the percentage of hemoglobin-containing K562 cells measured by benzidine staining as previously reported [9] and as the percentage of HL60 cells capable of reducing nitroblue tetrazolium (NBT) [1].

Assessment of toxicity

This study was performed by the method of Miller and Tainter. Acute toxicity was expressed in terms of the doselethal to 50% of the animals treated (LD_{50}) as determined in male Swiss (EOPS) albino mice weighing 18–20 g. Each derivative was injected intraperitoneally.

Results

Effects of butyric acid derivatives on growth and differentiation of K562 cells

Subtoxic concentrations of butyric acid derivatives that did not affect cell viability as assessed by the trypan blue exclusion test (>90% of cells were trypan blue-negative) induced K562 cell growth inhibition (Fig. 2A). At day 4 of culture, maximal cell growth inhibition (80–90%) without toxicity was obtained at a concentration of 1.25 m*M* for each derivative, whereas the parental compound, butyric acid, reached its maximal effect (95%) as soon as 1 m*M* was attained. At 1.25 m*M*, butyric acid exhibited a cytotoxic effect (>50% of cells died).

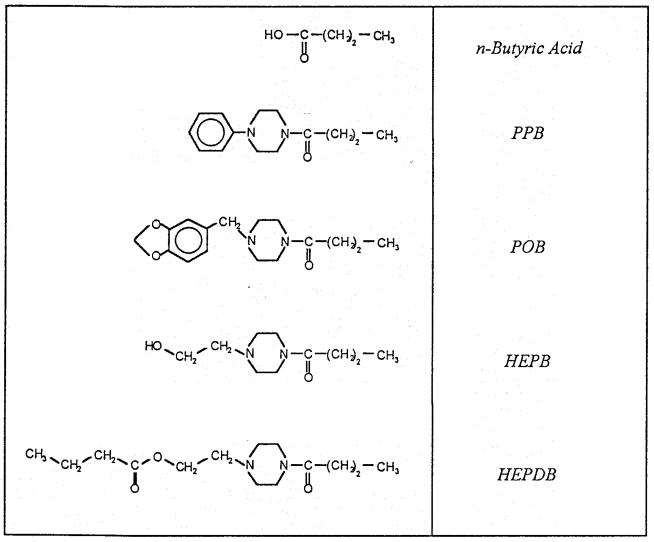

Fig. 1 Structures of the butyric acid piperazine derivatives synthesized

Surprisingly, butyrate derivatives did not exhibit the same effect on the appearance of hemoglobin-producing cells (Fig. 2B). A maximum of 55% differentiated cells was obtained with butyric acid, whereas the derivatives could be separated into two groups. POB and PPB derivatives had no action on the induction of cell differentiation, whatever the concentrations used (2–3% of cells were benzidine-positive versus 2% of uninduced cells), in spite of their marked antiproliferative effect. By contrast, as soon as 1 m*M* was reached, HEPB and HEPDB derivatives were more active (70% of cells were benzidine-positive) than the optimal inducing concentration of butyric acid (55% of cells were benzidine-positive at 0.5 m*M*).

Effects of butyric acid derivatives on growth and differentiation of HL60 cells

Figure 2C shows that butyric acid derivatives exhibited a concentration-dependent growth-inhibitory effect on HL60 cells at day 4. The maximal effect (75–90% growth inhibition) was measured at a concentration of 1.25 m*M* for each compound, except for HEPDB, which is cytotoxic at concentrations above 1 m*M*. In comparison, the optimal growth-inhibitory effect of butyric acid was obtained at the concentration of 0.5 m*M*. Above this value, butyric acid exhibited toxicity on this cell line (>50% of cells died at 0.75 m*M*).

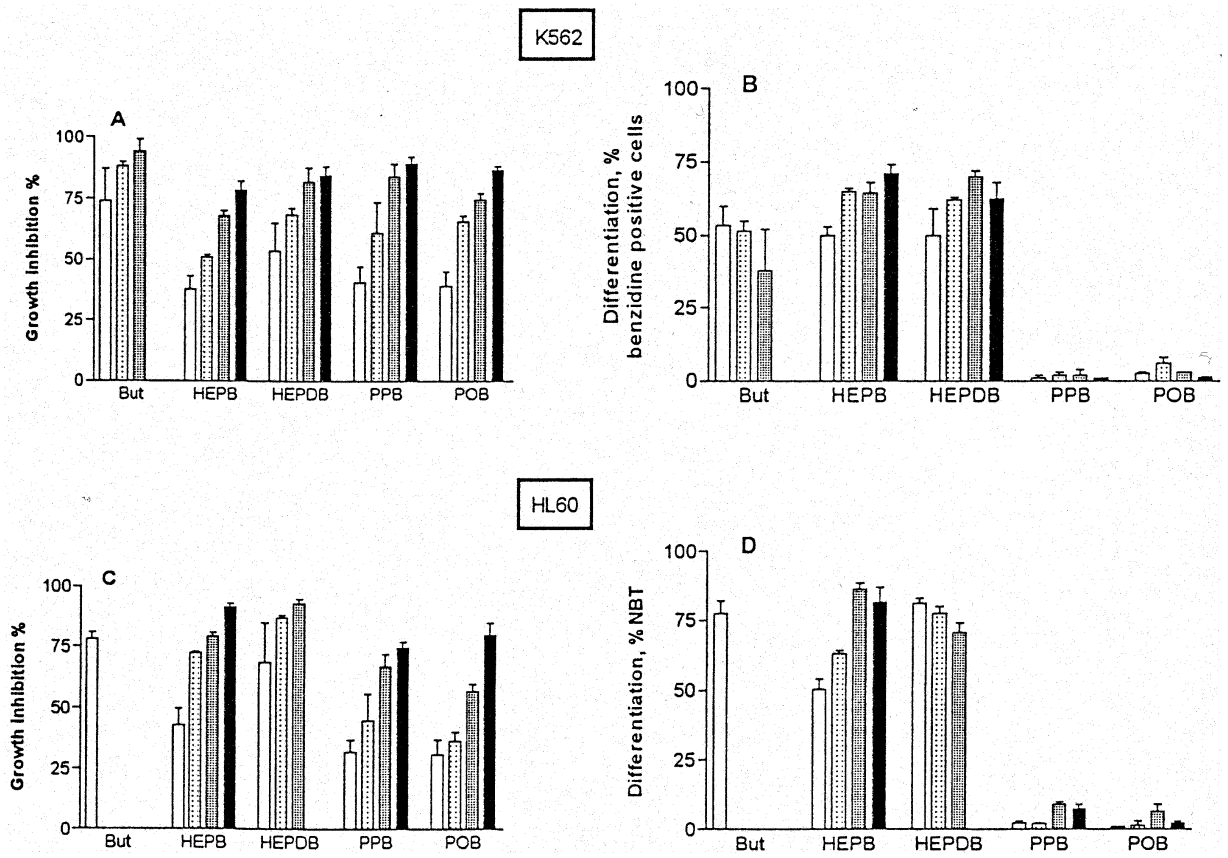

Fig. 2A–D Effects of butyric acid and its piperazine derivatives on the growth and differentiation of K562 and HL60 cells. **A, C** Cell growth inhibition and **B, D** differentiation induction were determined after 96 h of exposure to the butyrate derivatives at concentrations of 0.5 m*M* (□), 0.75 m*M* (▩), 1 m*M* (▨), and 1.25 m*M* (■). Results represent mean values for three independent experiments. The viability was ≥90% under all conditions

As observed with K562 cells, HEPB and HEPDB, but not POB or PPB, induced differentiation of HL60 cells (Fig. 2D). Indeed, HEPB (1–1.25 m*M*) and HEPDB (0.5–1 m*M*) induced about 85% of cells to become NBT-positive, similarly to butyric acid. By contrast, PPB and POB gave results similar to those obtained in uninduced cells (1–5%).

Assessment of toxicity

LD_{50} values were 365 ± 5 mg/kg for POB, 285 ± 8 mg/kg for PPB, >3 g/kg for HEPB, and 220 ± 10 mg/kg for HEPDB.

Discussion

Of the clinical trials of agents capable of inducing terminal differentiation of hematopoietic cells, very few seem to have a real in vivo potential. If all-*trans*-retinoic acid is henceforth employed in vivo in patients with acute promyelocytic leukemia [5], clinical use of butyric acid, in spite of its very promising effects, has been rapidly limited by its rapid clearance. The present study was directed at the development of nontoxic butyric acid analogues effective in acting on the coupling of proliferation and differentiation of leukemic cells. The results show that the piperazine derivatives tested react differently according to their molecular structure. The *n*-aliphatic derivatives HEPB and HEPDB induce differentiation and growth inhibition of both leukemic cell lines in a way equal or superior to the action of butyric acid. On the other hand, the POB and PPB derivatives, obtained by substitution of the piperazine portion with aromatic moieties, are not active on the differentiation pathway of either cell line, whereas they inhibit the proliferation of both. Development of such compounds is all the more interesting because the introduction of a piperazine core nevertheless permits very few toxic butyrate and dibutyrate derivatives to be obtained in vivo.

The exact mechanisms by which butyric acid and its derivatives activate differentiation programs remain unclear. Hyperacetylation of histones by the inhibition of desacetylase activity has been proven [25, 30] but cannot completely explain the biological effects of butyric acid. Many signaling pathways have particularly been described to explain the mechanisms of action of sodium butyrate, including tyrosine as well as serine/threonine kinases [26]. We have previously shown that anthracycline drugs, used as differentiating agents of erythroleukemic cells, can act at the transcriptional or posttranscriptional level by stimulat-

ing GATA-1 and NF-E2 transcription factors [2, 10, 12]; in a similar way, during hemoglobinization of K562 cells, butyric acid appears rapidly to increase mRNA levels of NF-E2 [7]. The existence of a transcriptional control of genes via butyrate-responsive promoters (BRPs) seems to be one of the most promising ways to develop related aromatic fatty acids that are capable of activating differentiation programs. One of these BRPs has been shown to up-regulate the transcription of the γ-globin gene in the presence of short-chain fatty acids [8]. It would be interesting to determine further whether the differentiating effects of butyric piperazine derivatives could involve the activation of BRPs. It would also be important to understand the molecular mechanisms by which HEPB and HEPDB act on both proliferation and differentiation, whereas POB and PPB exhibit only growth-inhibitory effects. These preliminary results may permit the development of more potent piperazine derivatives of butyric acid as anticancer agents.

Acknowledgements This study was supported by the Ligue Nationale contre le Cancer, comités de l'Aisne et de la Haute-Marne. R. G. is the recipient of a fellowship from the Ministère de l'Enseignement Supérieur et de la Recherche. The authors wish to thank Dr. Naïma Guedarri for her valuable advice, Dr. Roland Barret for helpful discussions, Laurence Van Gulick for technical assistance, and Nathalie Gillet for the grammatical proofing of the manuscript.

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