A Novel Approach for Nasopharyngeal Carcinoma Treatment Uses Phenylbutyrate as a Protein Kinase C Modulator: Implications for Radiosensitization and EBV-targeted Therapy

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ABSTRACT

Sodium phenylbutyrate (NaPB) represent a new nontoxic class of compounds with antiproliferative activities to different tumors and has been shown to modulate many gene expressions by inhibiting histone deacetylation and DNA methylation as the major mechanism. Butyrate and other protein kinase C (PKC) activators have been reported to be able to activate virus enzymes. The present work investigates whether NaPB has an antiproliferative effect or modulatory effects on EBV-associated nasopharyngeal carcinoma (NPC) and whether EBV thymidine kinase gene can be activated to make cells susceptible to ganciclovir (GCV) therapy. NaPB treatment displayed a dose- and time-dependent antiproliferative effect on the NPC cell line CNE2. Cell cycle analysis revealed an inhibitory effect of NaPB on G1/S-phase progression. Shortly after NaPB treatment, we found that PKC activity was activated rapidly but also decreased rapidly. Down-regulation of PKC-α and translocation of PKC-α from the cytosol to membrane were seen by Western blot. The decrease in PKC activity by NaPB corresponds to an enhanced response to radiation on CEN2 cells. Moreover, NaPB up-regulated EBV thymidine kinase activity to render EBV-associated Daudi cells susceptible to killing by GCV. Based on the observations of NaPB as a PKC modulator, the combination of NaPB, GCV, and radiation may provide a potential novel approach for treatment of EBV-associated NPC.

INTRODUCTION

NPC is a highly prevalent EBV-associated malignancy in Southern China (1). Radiotherapy and/or combined chemotherapy are the mainstay of treatment modalities. Because both modalities are usually associated with moderate complications, new approaches for treatment are needed.

In recent studies (2–13), NaPA has been observed to cause growth arrest and induce differentiation in many different cell lines from hematological malignancies to solid tumors including leukemia, neuroblastoma, rhabdomyosarcoma, breast carcinoma, hormone-refractory prostate adenocarcinoma, astrocytoma, glioblastoma, melanoma, and ovarian carcinoma. These data suggest that NaPA may represent a new nontoxic class of compounds with therapeutic potential in different cancer patients. NaPB, which is β-oxidized in vivo to NaPA (14), has been shown to be more potent than NaPA in inhibiting human prostate and ovarian carcinoma and does not smell disgusting like NaPA (7). Millimolar concentrations of NaPA or NaPB needed to inhibit tumors can be achieved clinically without significant adverse effects (2, 14).

Both NaPA and NaPB have the ability to turn on silent, redundant fetal and tumor suppressor genes and turn off oncogenes (2, 9, 11, 15–18). The mechanisms of NaPA and NaPB have been reported to inhibit histone deacetylation, DNA methylation, and protein isoprenylation (13, 19, 20). However, NaPB might act differently from NaPA. NaPB seems to be a more potent modulator of gene expression and acts in a manner analogous to butyrate (21). Sodium butyrate has been reported to induce differentiation like or augmented by TPA, a PKC activator (2, 22). Induction of virus enzymes by butyrate with or without phorbol esters has been reported by several authors. (23, 24) Because PKC activators can activate EBV TK expression, we ask whether NaPB, like other PKC activators, increases EBV TK expression to make EBV-associated tumors become a therapeutic target of GCV (25). The herpes virus TK phosphorylates GCV, which then inhibits the cellular DNA polymerase, leading to cell death. Without gene activation, most EBV in NPC exists in a latent state, with no response to GCV treatment.

The PKC pathway plays an important role in radiation-induced cell kill (26, 27). Because activated PKC is degraded quickly, treatment with PKC inhibitors or prolonged exposure to PKC activators results in PKC depletion and can enhance radiation-induced apoptosis. Therefore, if NaPB acts as a PKC activator, the combination of NaPB, GCV, and radiation may provide a potential novel approach for treatment of EBV-associated NPC.

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4 The abbreviations used are: NPC, nasopharyngeal carcinoma; NaPB, sodium phenylbutyrate; NaPA, sodium phenylacetate; PKC, protein kinase C; TPA, 12-O-tetradecanoylphorbol-13-acetate; GCV, ganciclovir; TK, thymidine kinase; TRAP, telomere repeat amplification protocol; RER, radiation enhancement ratio.
Table 1 Effects of NaPB on cell cycle distribution of CNE2 cells

<table>
<thead>
<tr>
<th>Exposure (h)</th>
<th>None</th>
<th>NaPB (5 mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>G0-G1</td>
<td>S phase</td>
</tr>
<tr>
<td>4</td>
<td>69</td>
<td>21.2</td>
</tr>
<tr>
<td>8</td>
<td>66.3</td>
<td>23.5</td>
</tr>
<tr>
<td>12</td>
<td>67.6</td>
<td>22.6</td>
</tr>
<tr>
<td>24</td>
<td>68.1</td>
<td>22.4</td>
</tr>
</tbody>
</table>

Values represent the mean of two experiments performed in duplicate; SD was less than 10%. Results are expressed as the percentage of cells in each phase of the cell cycle.

Flow Cytometry and Cell Cycle Analysis. After 4-, 8-, 12-, and 24-h treatments with 5 mM NaPB, cells were fixed in cold 100% ethanol for 1 h and stained with a PBS solution containing RNase A (100 μg/ml) and propidium iodide (25 μg/ml; Sigma) at room temperature for 1 h in the dark. DNA content was analyzed by using an Epics Profile Analyzer (Coulter Corp., Hialeah, FL).

Immunocytochemistry. After treatment with 5 mM NaPB for 12 h, cells were fixed and permeabilized by immersion in −20°C methanol/acetonone (1:1) for 10 min, treated with 3% H2O2 for 10 min, and incubated for 1 h at room temperature with 5 μg of PKC-α antibody (mouse antihuman antibody; Transduction Laboratories, Lexington, KY) and then incubated with a secondary antibody (goat antimouse antibody) conjugated to horseradish peroxidase (Transduction Laboratories) for 1 h. 3,3′-Diaminobenzidine substrate was added for 15 min. The slides were washed with water and stained with Mayer’s dye.

Western Blot Analysis. CNE2 cells treated with 5 mM NaPB for 12 h were homogenized in lysis buffer [4 mM EDTA, 2 mM EGTA, and 50 mM Tris-HCl (pH 7.4)] containing protease inhibitors (200 μg/ml each of phenylmethysulfonlfyl fluoride, leupeptin, and aprotinin) by sonication and then centrifuged for 1 h at 100,000 g (4°C). Supernatants were collected as the soluble (cytosol) fraction, and the pellets were rehomogenized by sonication in lysis buffer/protease inhibitor solution containing 1% Triton X-100. Homogenates were centrifuged, and the resulting Triton X-100-soluble fraction was collected as the membrane fraction. Both the cytosol fraction (35 μg) and the membrane fraction (45 μg) of samples were subjected to SDS-PAGE. Western blots with different PKC antibodies were detected using an enhanced chemiluminescence kit (Amersham).
PKC Activity Assay. After treatment with 5 mM NaPB for 5 min to 4 h, CNE2 cells were immediately placed in lysis buffer. Cytosol PKC activity was measured by using the MESACUP Protein Kinase Assay Kit (Medical and Biological Laboratory Co.). NaPB can modulate PKC activity (either activation or down-regulation) in different cell lines. After a short exposure to NaPB, PKC was activated immediately. With longer exposures, PKC activity was down-regulated.

EBV TK Activity Assay and GCV Cytotoxicity Assay. Because EBV exists in NPC cells as episomes that are easily lost in cell line passage, we used Daudi cells to test whether NaPB can up-regulate EBV TK activity. Daudi cells (EBV+) and HL-60 cells (EBV−) were pretreated with 2 mM NaPB for different time intervals from 12–48 h and then washed with fresh medium. An equal amount of the cytosol fraction was incubated with reaction buffer [50 mM Tris-HCl (pH 7.4), 1 mg/ml BSA, 3 mM creatine phosphate, 11.2 units/ml creatine phosphokinase, 0.1 mM [3H-8]GCV (specific activity, 13.5 Ci/mmol), 2.5 mM ATP, 2.5 mM MgCl2, 10 mM NaF, and 10 mM DTT] at 37°C for 30 min. The reaction mixtures were added on Whatman DE-81 ion exchange chromatography papers, which were then washed with 1.5 mM NH4COOH three times to remove the unphosphorylated form of [3H-8]GCV. The papers...
were air-dried and then incubated with a liquid scintillation mixture overnight. The phosphorylation form of [\(^{3}\)H-8]GCV bound on Whatman DE-81 ion exchange chromatography paper was measured by a LS6500 scintillation counter (Beckman). To test for GCV cytotoxicity, Daudi cells or HL-60 cells were preincubated or not preincubated with 2 mM NaPB for 48 h, and then 5 \times 10^4 cells/ml from each group were reseeded in media with or without GCV (20 \mu g/ml). Viable cell numbers were counted by trypan blue exclusion 3 days later.

RESULTS

The Effects of NaPB on Cell Growth and Telomerase Activity. The effects of different concentrations of NaPB on NPC cell line CNE2 were examined. As shown in Fig. 1, NaPB induced a dose- and time-dependent inhibition of cell proliferation. The cell cycle analysis of NaPB-treated cells is displayed in Table 1. After a 4-h exposure to 5 mM NaPB, the percentage of cells in the S phase of the cell cycle increased. However, after 8 h of treatment, the percentage of cells in the S phase began to decline, and after 24 h of treatment, a nearly complete blockage between G1 and S phase appeared. As shown in Fig. 2, loss of telomerase activity was detected by TRAP assay in CNE2 cells treated with NaPB, suggesting that differentiation might occur. Loss of telomerase activity in immortalized cells has been reported to correlate with cellular differentiation (30). Gross phenotypic changes were observed after treatment with 5 mM NaPB for 3 days. CNE2 cells appeared to became larger, longer, and flatter, and the nucleus:cytoplasm ratio decreased. The differentiation-like cells did not pile up and just formed one layer with a contact inhibition pattern. Although morphological alterations were seen after NaPB treatment, no definitive markers can be used for confirmation of differentiation.

NaPB Can Modulate PKC Activity. As shown in Fig. 3, an initial increase of in PKC activity is induced by a short exposure (5–30 min) to NaPB. The PKC activity was then down-regulated by persistent NaPB treatment (NaPB treatment of \(\geq 1\) h). The initial increase followed by a decline in PKC activity after short exposure to NaPB was a common phenomenon in several different cell lines (Fig. 3). The modulation effect on PKC activities by NaPB seems to be a universal effect in different cell types. To demonstrate that NaPB treatment could degrade and translocate certain PKC isoforms, we use immunocytochemistry and Western blot to demonstrate PKC-\(\alpha\) down-regulation by NaPB and translocation of PKC-\(\alpha\) from the cytosol to membrane (Fig. 4, A and B). Taken together, NaPB is likely to be a PKC activator, but prolonged treatment will result in PKC inhibition due to PKC depletion.

NaPB Increases Radiation Sensitivity of NPC Cells. As shown in Fig. 5, a low dose (noncytotoxic concentration) of NaPB (1 or 2 mM NaPB) administered 60 min before
radiation increased the RER at 10% survival to 1.3 and 1.6, respectively. These data suggest that NaPB acted as a radiosensitizer.

NaPB Can Up-Regulate EBV TK Activity to Make EBV-associated Tumors Susceptible to GCV Killing. GCV can be phosphorylated by EBV TK to inhibit cellular DNA polymerase, causing cell death in the S phase of cell cycle. We took advantage of the reversibility of growth inhibition by changing to fresh media after 48 h of low-dose (2 mM) NaPB treatment. TK activity was detected by measuring the level of phosphorylation of [3 H-8]GCV. As shown in Fig. 6A, EBV TK activity was up-regulated by NaPB treatment. There is a clearly time-dependent effect of NaPB on the up-regulation of EBV TK activity. The subsequent addition of GCV killed EBV-1 Burkitt’s lymphoma cells but not EBV-2 HL-60 leukemia cells (Fig. 6B).

DISCUSSION

In this study, we have demonstrated that NaPB has an antitumor effect on NPC cells in vitro and that the PKC pathway is involved. This result was consistent with previous reports that NaPB could induce differentiation and apoptosis in many other cell lines (2, 11). The antiproliferative effect of NaPB is dose and time dependent, and a longer exposure time is necessary for low-dose NaPB to produce the same results as high-dose NaPB. Although exposure to a higher concentration of NaPB (5 mM) for 72 h is necessary to demonstrate the inhibition effect of telomerase activity and gross morphology changes in CNE2 cells, a lower dose (1–2 mM) or a shorter exposure time is sufficient to modulate PKC activity, up-regulate EBV TK activity, and observe radiosensitization activity. Our study provides a potentially applicable strategy for the combination of NaPB in a clinically achievable millimolar range with radiotherapy and GCV for NPC treatment.

Our study indicates, for the first time, that some of the versatile antitumor abilities of NaPB may be mediated by a common signal pathway via activation of certain PKC isoforms such as PKC-α (Fig. 3). Many studies have provided evidence that the PKC pathway is linked to cell proliferation, differentiation, and apoptosis (31, 32). PKC-α activation can enhance tumor cell differentiation and decrease proliferation (33). An increase in PKC activity followed by a decline in PKC activity and down-regulation of PKC can be seen after initial and prolonged treatment with NaPB, respectively (Fig. 3). This could also explain why treatment with protracted exposure to PKC activators or with PKC inhibitors can be observed to have similar effects (2, 34). We regard NaPB, like TPA, as a PKC activator that activates PKC quickly and consumes PKC quickly. In fact, preincubation with H7, a PKC inhibitor, was found to partially inhibit the antiproliferative effect of NaPB (data not shown).

The inhibition of telomerase activity by PKC inhibitors in human NPC cells was first examined by Ku et al. (35). They examined the effects of cell cycle blockers, DNA-damaging agents, topoisomerase inhibitors, and protein kinase inhibitors on telomerase activity in cultured NPC-076 cells. They found that only PKC inhibitors were effective in producing an inhibition of telomerase activity. NaPB produced a profound inhibition of telomerase activity. Sharma et al. (36) reported that down-regulation of telomerase activity was found to be a general response to the induction of differentiation. On the basis of these results and our evidence that NaPB inhibits PKC after a few hours of exposure, we agree that PKC is involved in the regulation of telomerase activity.

Therefore, the modulation effects of NaPB on cellular PKC activities can be used to enhance the radiation effect because down-regulation of PKC activity has been reported to increase radiosensitivity (26, 27). As shown in Fig. 3, a 60-min exposure to NaPB decreased PKC activity, and a radiosensitization effect was found at that time point. Miller et al. (37) demonstrated a radiosensitization effect by NaPB pretreatment, but the effect is dependent on exposure time. They found that the radiosensit-
five experiments performed in triplicate. Viable cells were counted 3 days later.

### Fig. 6

Up-regulation of EBV TK and increase in GCV sensitivity of EBV-associated tumor cells by NaPB. Daudi cells (EBV+) and HL-60 cells (EBV–) were pretreated with 2 mM NaPB for different time intervals from 12–48 h and then washed with fresh medium. A. TK activity was detected by measuring the level of phosphorylation of [3H]-8GCV. EBV TK activity was up-regulated by NaPB in a time-dependent manner. B. Daudi cells (EBV+) and HL-60 cells (EBV–) were pretreated with 2 mM NaPB for 48 h and then plated to another flask without NaPB. Five × 10⁶ cells/ml from each group were seeded with or without the addition of GCV (20 μg/ml) for a 3-day culture. Viable cells were counted 3 days later. Bars, the mean of three different experiments performed in triplicate.

TK activity but also acted synergistically with GCV to inhibit cell proliferation and decrease cell viability in our Daudi cell model. The role of NaPB as a PKC modulator and gene modulator raises the therapeutic possibility of targeting EBV to selectively destroy EBV-associated tumor cells. According to our result in Fig. 6B, the GCV sensitivity of Daudi cells treated with NaPB was so high that a bystander effect might be involved, and we are now exploring this possibility.

In conclusion, we have demonstrated an inhibition effect on cell proliferation, cell cycle progression, and telomerase activity in CNE2 cells by NaPB treatment. We found that down-regulation of PKC activity is involved as one of the mechanisms of action of this interesting compound. NaPB in a clinically achievable concentration may serve as a good radiosensitizer and sensitizes EBV-associated cells to GCV. In view of NaPB as a relatively nontoxic agent for cancer treatment, the combination of NaPB, GCV, and radiotherapy may produce a therapeutic benefit on EBV-associated tumors such as Hodgkin’s lymphoma, lymphoepithelioma of parotid gland, and Burkitt’s lymphoma as well as NPC.

### REFERENCES


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