

# Prevention and Inhibition of Nasopharyngeal Carcinoma Growth by Antiviral Phosphonated Nucleoside Analogs<sup>1</sup>

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## ABSTRACT

Nasopharyngeal carcinoma (NPC) is universally associated with EBV infection. We have shown that the phosphonated nucleoside analog, (S)-1-[3-hydroxy-2-(phosphonyl-methoxy)-propyl]cytosine (HPMPC) strongly inhibits growth of NPC xenografts in nude mice by causing apoptosis (J. Neyts *et al.*, *Cancer Res.*, 58, 384-388, 1998). We, therefore, tested two additional members of this drug family that have different degrees of antiviral activity, 9-[2-(phosphonomethoxy)ethyl]adenine (PMEA) and 9-2-(R)-(phosphonomethoxy)propyladenine (PMPA). Intratumoral injection of PMEA (75  $\mu$ l of 2% solution) in C15 NPC xenografts, which are latently infected with EBV, slowed tumor growth moderately, whereas PMPA (75  $\mu$ l of 2% solution) slowed tumor growth only marginally. Compared with the previous results showing complete regression of tumor, PMEA had less antitumor effect than HPMPC, and PMPA had the least. After 4 weeks of preventive treatment, tumors formed in 12.5, 50, and 100% of mice treated with HPMPC, PMEA, and PMPA, respectively, in contrast to the development of tumors in all of the PBS-treated control mice. We also investigated the effect of each drug on the EBV-positive epithelial cell line NPC-KT *in vitro*. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay showed inhibition of growth of NPC-KT cells by HPMPC and PMEA, but not by PMPA, which correlates with the results observed in tumor xenografts. Growth inhibition was attributable to induction of apoptosis in NPC-KT cells as indicated by a DNA fragmentation assay. Cleavage of poly(ADP-ribose) polymerase after treatment of NPC-KT cells with HPMPC was observed, which suggested that the apoptosis may be mediated by caspase(s). The apoptotic effects of the drugs are independent of any effects on EBV DNA polymerase, which is not expressed in these latently infected NPCs. These results suggest that HPMPC as well as PMEA could provide an adjunctive treatment for NPC.

## INTRODUCTION

The acyclic nucleoside phosphonates [HPMPC<sup>3</sup> (cidofovir), PMEA (adefovir), and PMPA] have proved to be effective in cell culture systems, animal models, and clinical studies against a wide variety of DNA virus and retrovirus infections. HPMPC has efficacy against herpesvirus (herpes simplex virus types 1 and 2, varicella-zoster virus, cytomegalovirus, EBV, human herpesvirus 6, 7, and 8), polyomavirus, papillomavirus, adenovirus, and poxvirus (vaccinia and molluscum contagiosum viruses) infections; PMEA has efficacy against herpesvirus, hepadnavirus (human hepatitis B virus), and retrovirus (human immunodeficiency virus types 1 and 2, and simian and feline immunodeficiency viruses) and EBV infections; and PMPA has efficacy against both hepadnavirus and retrovirus infections (1-3). These molecules are characterized by a stable phosphonate linkage between the acyclic nucleoside and the phosphate

moiety. In contrast to antiviral drugs such as acyclovir and ganciclovir, these drugs bypass the first phosphorylation step by herpesvirus-encoded kinases. Cellular kinases phosphorylate these drugs, and the diphosphorylated metabolite selectively inhibits the viral DNA polymerases required for viral replication (4).

Quite distinct from these antiviral effects, two of these drugs have proved to be effective for tumors in which virally encoded DNA polymerases are not expressed. HPMPC has a dramatic inhibitory effect on severe recurrent laryngeal papillomatosis caused by human papillomavirus (5, 6). HPMPC has also been reported to inhibit growth of xenografts in nude mice that were derived from papillomavirus-positive human cervical cancer cells (7). Moreover, clinically, HPMPC in a gel, applied topically, inhibits cervical dysplastic lesions (8). Finally, PMEA has been shown to inhibit the growth of choriocarcinoma in rats (9).

NPC, which is endemic in southern China and Taiwan, is universally associated with EBV infection (10). The EBV genomes in NPC are monoclonal (11), which suggests that NPC develops from a single EBV-infected cell rather than a secondary infection of a proliferating tumor and is consistent with a causal role for EBV in NPC. In NPC, EBV infection is mainly latent, and the tumors contain episomal viral DNA rather than linear genomes. Episomal DNA is replicated by host DNA polymerase, whereas the linear genomes produced in cytolytic infection are replicated by EBV DNA polymerase. NPC is usually treated by irradiation and chemotherapy.

We have demonstrated that HPMPC has pronounced apoptotic effects against xenografts of NPC latently infected with EBV and grown in nude mice (12). Here we report that two other less potent members of the phosphonated nucleoside analog family, PMEA and PMPA, also have activity, although less so, against the growth of NPC xenografts. We also demonstrate growth inhibition by induction of apoptosis by HPMPC in an EBV-positive epithelial cell line derived from NPC.

## MATERIALS AND METHODS

**NPC Xenografts Grown in Nude Mice.** The NPC xenograft C15 was passaged in nude mice as before (12).

**Intratumoral Treatment.** HPMPC was prepared as a 2% solution in PBS. PMEA and PMPA were prepared as 2% solutions in distilled water and were sterilized through filtration. The NPC xenograft C15 was grown s.c. until the size of the tumor reached 0.5-1.0 cm<sup>3</sup>, followed by intratumoral injection with 75  $\mu$ l of drug or PBS per day for 28 consecutive days at almost the same dose used previously (12).

**Preventive Protocol.** Two days after s.c. implantation of the C15 tumor, each mouse was treated daily systemically with 75  $\mu$ l of drug or of PBS by s.c. inoculation. Tumor formation was evaluated after 4 weeks' treatment.

**Salvage Treatment with HPMPC.** After 4 weeks under the preventive protocol with PMEA or PMPA, tumors formed in some mice (as described in "Results"). These tumors were inoculated directly with 75  $\mu$ l of HPMPC or PBS daily. Tumor volume was evaluated weekly during 21 days of treatment.

**Cells.** NPC-KT (13) is an EBV-positive epithelial cell line derived from fusion between EBV-negative nasopharyngeal epithelial Ad-AH cells and EBV-positive NPC tissue. Cells were cultured in DMEM supplemented with 10% fetal bovine serum and antibiotics.

**MTT Assays.** NPC-KT cell proliferation was analyzed with the MTT assay (14). Cells were treated with PBS or each drug (1 mM); then the MTT assay was performed on days 1, 3, and 5. Administration of PBS or each drug was

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<sup>3</sup> The abbreviations used are: HPMPC, (S)-1-[3-hydroxy-2-(phosphonyl-methoxy)-propyl]cytosine; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NPC, nasopharyngeal carcinoma; PARP, poly(ADP-ribose) polymerase; PMEA, 9-[2-(phosphonomethoxy)ethyl]adenine; PMPA, 9-2-(R)-(phosphonomethoxy)propyladenine; EBER, EBV-encoded small RNA.

done once at the beginning of culture. Spectrophotometric absorbance at 595 nm (for formazan dye) was measured with absorbance at 655 nm for reference.

**Western Blotting and DNA Fragmentation Assays.** NPC-KT cells were treated with PBS or HPMPC (1 mM) for 24 h and 96 h. PARP expression was analyzed with rabbit polyclonal anti-PARP antibody by Western blotting as described previously (14). DNA fragmentation was also analyzed as described previously (15).

## RESULTS

**Effect of PME A and PMPA on Growth of NPC Xenografts.** We have shown that HPMPC has a pronounced inhibitory effect on the growth of NPC xenografts C15, C17, and C18, after intratumoral and systemic injection (12). To confirm and extend these results, we tested the related drugs, PME A and PMPA, on the growth of C15 NPC xenografts. Intratumoral injection with PME A produced a moderate inhibitory effect on the growth of C15 xenografts, whereas treatment with PMPA showed only a marginal inhibitory effect (Fig. 1), consistent with the lesser antiviral potencies of these two drugs.

**Prevention of Tumor Formation.** Systemic administration of drug was begun within 2 days after tumor implantation to see whether either drug could prevent tumor formation. After 4-week treatment with PBS or drug, tumor formation was evaluated (Table 1). In all seven mice treated with PBS, obvious tumors developed ranging in size from 6 to 11 mm in maximum diameter. In contrast, in only one of eight mice treated with HPMPC did a small tumor (<5 mm) develop. In addition, all eight mice treated with HPMPC for 4 weeks were observed for another 4 weeks without any treatment, which resulted in the appearance of a small tumor (<5 mm) in another mouse. The initial tumor dating from 4 weeks earlier remained the same size. In two of four mice treated with PME A and all of the four mice treated with PMPA, tumors developed after 4 weeks of administration of the drugs. Thus, it was possible to prevent tumor formation by treatment with HPMPC and also PME A. These data are in agreement with the inhibition of tumor growth produced by inoculation of drug directly into the tumors.

**Salvage from Failed Preventive Treatment.** Because the antitumoral activity of HPMPC is pronounced, we attempted to rescue mice

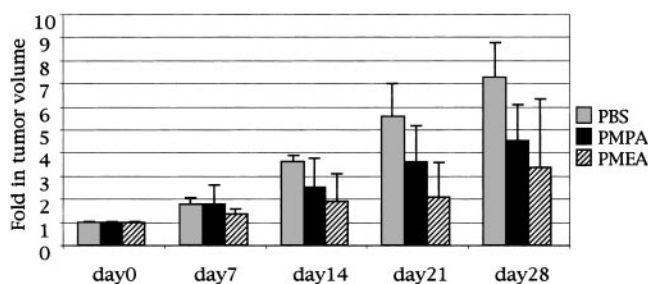


Fig. 1. PME A and PMPA inhibit the growth of C15 NPC xenografts in nude mice. Intratumoral treatment with PBS or either drug (75  $\mu$ l of 2% solution) was initiated when tumor volume had reached a size between 0.5 cm<sup>3</sup> and 1.0 cm<sup>3</sup>, and continued for 28 days. Tumor volume was measured every week. Each group contained four mice. Change in tumor volume is shown with SD.

Table 1 Prevention of tumor formation with immediate treatment after tumor implantation

Treatment	No. of mice treated	No. of mice with tumor <sup>a</sup>	No. of mice with tumor <sup>b</sup>
PBS	7	7	7
HPMPC	8	1	2
PME A	4	2	ND <sup>c</sup>
PMPA	4	4	ND

<sup>a</sup> Occurrence of tumor after 4 weeks preventive treatment.

<sup>b</sup> Occurrence of tumor after 4 weeks preventive treatment plus 4 weeks with no treatment.

<sup>c</sup> ND, not done.

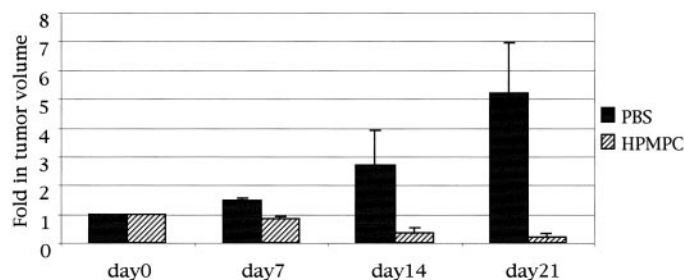


Fig. 2. HPMPC inhibited the growth of tumors that had failed preventive treatment with PME A or PMPA. Tumor formation occurred in four mice with PME A preventive treatment and two mice with PMPA preventive treatment (Table 1). After 4 weeks of the preventive regimen, two of four mice previously treated with PMPA were treated with PBS, and the other four mice previously treated with either PME A or PMPA were intratumorally inoculated with HPMPC (75  $\mu$ l of 2% solution) for rescue. Treatment continued for 21 days, and tumor volume was measured every week. Changes in tumor volume are shown with SDs.

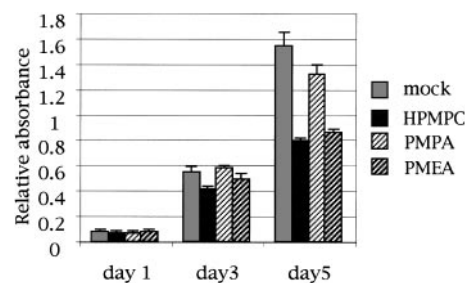


Fig. 3. MTT assays. Five-thousand NPC-KT cells were cultured with either PBS or drug (1 mM), followed by measurement of absorbance at days 1, 3, and 5. Relative absorbance with SD is shown.

in which the prevention of tumor formation by either PME A or PMPA had failed. Treatment with HPMPC dramatically inhibited the growth of all tumors that had continued to grow under the prevention protocol, which suggests that resistance to HPMPC did not develop after exposure to PME A and PMPA (Fig. 2).

**Induction of Apoptosis by HPMPC in NPC-KT Cells.** To find whether the inhibitory effect of HPMPC could be demonstrated in an EBV-positive NPC cell culture system, we tested the effect of the drug on NPC-KT cells. We first used the MTT assay to assess the effect of HPMPC on cell proliferation. Treatment of NPC-KT cells with HPMPC markedly inhibited the growth of the cells (Fig. 3) by day 5, although the effect was not as great as with NPC xenografts grown in nude mice. PME A also inhibited cell proliferation whereas PMPA had little effect. To confirm whether the inhibition of growth produced by HPMPC was attributable to apoptosis, a DNA fragmentation assay was performed. Treatment with HPMPC for 96 h induced marked DNA fragmentation; however, treatment for 24 h did not produce detectable fragmentation (data not shown). In contrast to these *in vitro* studies, HPMPC produced striking apoptosis in xenografts within 2 days, as indicated by the TUNEL assay (12). Apoptosis was apparently mediated by the activation of caspases, because cleavage of PARP from  $M_r$  116,000 to 86,000 was detected in NPC-KT cells treated with HPMPC for 96 h compared with cells treated with PBS (Fig. 4).

## DISCUSSION

This study was designed to confirm and extend previous observations of striking antitumoral effects of HPMPC in NPC (12). We focused on three aspects: (a) the antitumoral effect of related drugs; (b) preventive treatment against NPC and rescue for failed cases; and (c) application of *in vivo* results to EBV-positive epithelial cells *in vitro*.

Like HPMPC, PME A has antiviral effects against herpesviruses and

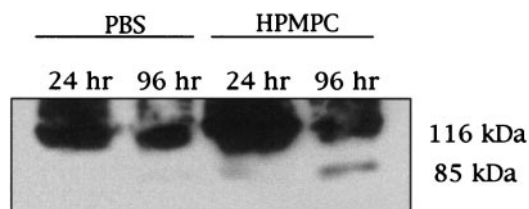


Fig. 4. Western blotting for PARP. NPC-KT cells were cultured with either PBS or HPMPC (1 mM); then cells were collected at day 1 and day 4. One hundred  $\mu$ g of protein were analyzed for PARP expression. Bands at  $M_r$  116,000 and at  $M_r$  85,000 show uncleaved and cleaved forms of PARP, respectively. *kDa*,  $M_r$  in thousands.

human immunodeficiency virus (16). PMPA also has antiviral effects against some retroviruses (16). Therefore, although the antitumor effect of HPMPC on NPC is not dependent on its antiviral DNA polymerase activity, antitumor effects of PMEA and PMPA would be possible and expected, even though these drugs are generally less potent than HPMPC (2, 3). PMEA had an obvious inhibitory effect on growth of C15 tumors, whereas PMPA had a marginal effect. However, in contrast to treatment with HPMPC, which produced tumor regression (12), the NPC xenografts continued to grow during treatment with PMEA or PMPA. Thus, the antitumor effect of HPMPC on C15 tumors is considerably greater than that of PMEA or PMPA.

Interesting findings in this study are that HPMPC can both prevent C15 tumor outgrowth and rescue mice that had failed treatment with PMEA and PMPA. These results reflect the relative direct antitumor effects of the three drugs when they were injected into the tumors (Figs. 1 and 2). Because recurrence after primary treatment is one of the most important factors affecting prognosis of NPC, this study raises the possibility that HPMPC could be used prophylactically to prevent tumor recurrence.

Furthermore, because later treatment with HPMPC of tumors that had survived exposure to PMEA or PMPA completely suppressed their growth, these results also suggest that the previous treatment did not cause resistance of the C15 tumors to HPMPC.

Inhibition of proliferation of NPC-KT cells by HPMPC was confirmed in a cell culture system, but MTT assays did not show complete inhibition. This result may reflect differences in the protocols, because HPMPC was injected daily into C15 tumors, whereas NPC-KT cells were cultured with only one dose of HPMPC in the medium. Also, *in vivo* there are many kinds of cells in addition to tumor cells that may contribute to the inhibition of growth produced by HPMPC.

In our previous report, histological examination and terminal deoxynucleotidyl transferase-mediated dUTP nicked-end labeling in C15 tumors revealed a large increase in apoptotic cells after intratumoral injection with HPMPC (12). The HPMPC-induced apoptosis was not p53-dependent because NPCs both with and without p53 mutations underwent apoptosis to the same extent (12). Previous studies have shown the correlation between PARP cleavage and caspase activation (17–19). The detection of PARP cleavage after treatment suggests that the apoptosis induced by these drugs may be mediated by caspases.

It is also possible that HPMPC affects viral expression and reduces viral suppression of apoptosis. We have shown previously (12) the loss of EBV expression in treated animals. This effect may contribute to apoptosis, because the EBVs contribute to the malignant potential of Burkitt's lymphoma, another major EBV-associated malignancy, through inhibitory effects on apoptosis (20, 21). Furthermore, HPMPC may affect expression of viral proteins such as LMP1, which is expressed in NPC, and protects against apoptosis through up-regulation of the antiapoptotic *bcl-2* gene (22) and A20 (23). However, the regression of tumor growth and induction of apoptosis in nude mice by HPMPC was observed not only in C15 tumors, which are LMP-1-positive xenografts, but also in C17 and C18 tumors, which are EBV-positive but LMP-1 negative, at

least at the protein level. Therefore, although HPMPC could apparently affect expression of LMP-1, the viral protein may not be involved in HPMPC-induced apoptosis.

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