

Inhibition of Fibroblast Growth Factor-2-induced Vascular Tumor Formation by the Acyclic Nucleoside Phosphonate Cidofovir¹

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ABSTRACT

Cidofovir [(S)-HPMPC; (S)-1-(3-hydroxy-2-phosphonylmethoxypropyl)cytosine] is an antiviral drug that has been approved for the treatment of cytomegalovirus retinitis in AIDS patients. Cidofovir also possesses potent inhibitory activity against various human papillomavirus-induced tumors in animal models and patients. In addition, cidofovir inhibits the development of murine polyomavirus-induced hemangiomas in rats by an as-yet-uncharacterized, antiviral-independent mechanism. Here we report the inhibitory effect of cidofovir on the development of virus-independent vascular tumors originated by basic fibroblast growth factor (FGF2)-overexpressing endothelial cells (FGF2-T-MAE cells). *In vitro*, cidofovir was cytostatic to FGF2-T-MAE cells at a 50% cytostatic concentration of 6.7 $\mu\text{g/ml}$. Cidofovir concentrations >25 $\mu\text{g/ml}$ resulted in cytotoxicity because of induction of apoptosis. Cidofovir did not affect FGF2-T-MAE cell sprouting in three-dimensional fibrin gel and morphogenesis on Matrigel at noncytotoxic concentrations. *In vivo*, cidofovir (100 $\mu\text{g/egg}$) completely suppressed hemangioma formation on the chick chorioallantoic membrane (CAM) induced by intra-allantoic injection of FGF2-T-MAE cells, without affecting the formation of normal CAM vessels. Accordingly, cidofovir applied locally at 200 $\mu\text{g/disc}$, reduced neovascularization on the CAM by only 35%. Intratumoral or systemic administration of cidofovir caused a significant inhibition of the growth of s.c., i.p., or intracerebral FGF2-T-MAE xenografts in nude mice and severe combined immunodeficient mice. Drug-induced apoptosis was observed in FGF2-T-MAE tumors as soon as 2 days after the beginning of treatment. In conclusion, cidofovir appears to inhibit the growth of endothelium-derived tumors via induction of apoptosis without exerting a direct antiangiogenic activity.

INTRODUCTION

Cidofovir³ [(S)-HPMPC] belongs to a class of acyclic nucleoside phosphonates; these are structural analogues of nucleotides consisting of an acyclic nucleoside moiety to which a phosphonate group has been attached through a stable P-C linkage (1). Acyclic nucleoside phosphonates are active *in vitro* and *in vivo* against a broad range of DNA viruses including herpes viruses, poxviruses, hepadnaviruses, adenoviruses, and papovaviruses (1–6). Cidofovir has been approved for the treatment of cytomegalovirus retinitis in AIDS patients. Furthermore, topically or systemically administered cidofovir has been explored for the treatment

of infections with other herpesviruses, poxviruses, adenoviruses, and human polyomaviruses and HPVs (7–12).

After cellular uptake, presumably via fluid-phase endocytosis (13), HPMPC is intracellularly converted to three metabolites: HPMPC monophosphate, HPMPC diphosphate (HPMPCpp), and HPMPCp-choline (14). The choline adduct has a half-life of 87 h and may serve as a reservoir from which the active metabolite HPMPCpp can be continuously generated, thus explaining the prominent antiviral activity of HPMPC upon infrequent dosing (15, 16). The antiviral effect of HPMPC results from a selective inhibition of the viral DNA polymerase by its diphosphate metabolite. HPMPCpp acts as a competitive inhibitor and/or an alternative substrate with respect to the normal substrate dCTP during the DNA polymerase process (17).

In addition to its antiviral activity, cidofovir has been shown recently to elicit a potent antitumor activity. Intratumoral therapy with cidofovir proved effective in the treatment of recurrent, HPV-associated, laryngeal papillomatous lesions in patients (18). Furthermore, topically applied cidofovir has caused complete regression of severe, relapsing penile, perigenital/intraanal, or cervical/vulvar condylomata and grade III cervix intraepithelial neoplasia associated with HPV (19, 20). Topical cidofovir also led to successful resolution of severe lesions caused by the molluscum contagiosum virus in immunodeficient patients without any signs of systemic side effects (21, 22). In contrast, intralesional injections of cidofovir did not afford a protective effect in one case of classical Kaposi's sarcoma (23).

The major side effect of systemically administered cidofovir, *i.e.*, nephrotoxicity, can be overcome by prehydration and concomitant administration of probenecid (24), an inhibitor of organic anion transport, which interferes with the transporter-mediated tubular uptake of cidofovir (25). No side effects have been observed after topical or intralesional administration of the drug (18).

In animal models, intratumoral administration of cidofovir resulted in regression of HPV-16-positive, human cervical carcinomas (SiHa; Ref. 26) and established NPC xenografts in nude mice (27). After a 2–6-day treatment period with cidofovir, widespread apoptosis was observed in the NPC xenografts. Thus, cidofovir induces cell death through apoptosis in EBV-transformed epithelial cells. Finally, we recently presented a novel animal model for hemangiomas induced by the murine PyV (28) in which cidofovir caused complete protection at clinically achievable doses (29). Thus far, all antitumoral data with cidofovir were generated in animal studies with virus-transformed xenografts or clinical studies with HPV-associated tumors, which are in line with a presumable antiviral mode of action (17). However, the potent inhibitory activity of cidofovir against PyV-induced hemangiomas in rats could not be explained by an antiviral mechanism (29).

A growing tumor needs an extensive network of capillaries to provide nutrients and oxygen (30). Angiogenesis is a complex process involving extensive interplay between cells, soluble factors, and extracellular matrix components. The angiogenic basic fibroblast growth factor (FGF2) has been shown to induce endothelial cell proliferation, chemotaxis, and protease production *in vitro* and angiogenesis *in vivo* (31, 32). Moreover, FGF2 has been implicated in the pathology of several angiogenic diseases, including KS and hemangiomas (33, 34). Endothelial cells overexpressing FGF2 (FGF2-T-MAE cells) have

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³ The abbreviations used are: cidofovir, (S)-HPMPC [(S)-1-(3-hydroxy-2-phosphonylmethoxypropyl)cytosine]; HPV, human papillomavirus; ara-C, cytarabine; CAM, chorioallantoic membrane; NPC, nasopharyngeal carcinoma; CC₅₀, 50% cytostatic concentration; FGF2, basic fibroblast growth factor; SCID, severe combined immunodeficient; *i.t.*, intratumoral; KS, Kaposi's sarcoma; MAE, mouse aortic endothelial; PyV, murine polyomavirus; PCNA, proliferating cell nuclear antigen; TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling; MDD, mean day of death.

been shown to possess tumorigenic activity in nude mice, giving rise to highly vascularized lesions that histologically resemble KS (35, 36). When inoculated into the allantoic sac of a chick embryo, FGF2-*T*-MAE cells cause an increase in vascular density and the formation of hemangiomas on the CAM (36, 37).

The present study was designed to gain further insight into the mechanism of antitumor activity of cidofovir by using the virus-independent vascular tumor model represented by FGF2-*T*-MAE cells. Therefore, we evaluated the effect of cidofovir on angiogenesis and the growth of FGF2-overexpressing endothelial cells *in vitro* and *in vivo*. The results demonstrate for the first time the antitumor activity of cidofovir against lesions that are not induced by viruses.

MATERIALS AND METHODS

Materials. Cidofovir (Vistide; Fig. 1A) was kindly provided by Gilead Sciences (Foster City, CA). Ganciclovir (Cymevene) was obtained from Roche (Brussels, Belgium), and cytarabine (ara-C; Cytosar) was from Upjohn (Puurs, Belgium).

Cell Cultures. FGF2-transfected MAE cells (pZipbFGF2-MAE) express high levels of the M_r 18,000, M_r 22,000, and M_r 24,000 molecular weight isoforms of FGF2 (36). After injection in nude mice, these cells induced the formation of vascular lesions from which FGF2-*T*-MAE cells could be isolated. When reinjected in nude mice, FGF2-*T*-MAE cells induced the formation of tumors more rapidly than the parent pZipbFGF2-MAE cells (35). FGF2-*T*-MAE cells were grown in DMEM (Life Technologies, Inc., Rockville, MD) supplemented with 4 mM glutamine (Life Technologies, Inc.) and 10% FCS (Integro, Zaandam, the Netherlands).

Cell Proliferation Assays. For measurement of cell growth, FGF2-*T*-MAE cells were seeded at 20,000 cells/cm² in DMEM with 10% FCS. After 24 h, the medium was replaced, and cidofovir was added. The cell cultures were

incubated for 6 days, trypsinized, and counted. To examine the reversibility of the effect of cidofovir on FGF2-*T*-MAE cell proliferation, cells were washed 2 days after addition of cidofovir and further incubated in the absence of the compound for 4 days.

Detection of Apoptosis. To detect apoptosis in individual cells, FGF2-*T*-MAE cells were seeded in four-well chamber slides (Nunc, Naperville, IL) at 50,000 cells/cm². Subconfluent cells were treated with cidofovir at 10, 25, 50, 100, or 250 μg/ml for 1, 2, 3, or 4 days. At the indicated time points, cells were fixed in 4% paraformaldehyde in PBS for 1 h at room temperature, rinsed with PBS, and permeabilized for 2 min on ice in 0.1% Triton X-100 in 0.1% sodium citrate. Apoptotic cells were visualized by means of the *In Situ* Cell Death Detection kit (Boehringer Mannheim, Mannheim, Germany). This kit relies on the use of terminal deoxynucleotidyl transferase, which catalyzes the polymerization of fluorescein-labeled nucleotides to free 3'-hydroxyl residues of DNA fragments, generated by endonucleases during apoptosis. Fluorescein-labeled DNA strand breaks in apoptotic cells could subsequently be detected with a fluorescence microscope.

CAM Assay. The *in vivo* CAM angiogenesis model was performed as described by Maragoudakis *et al.* (38). Briefly, fresh fertilized eggs were incubated for 3 days at 37°C when 3 ml of albumen were removed (to detach the shell from the developing CAM), and a window was opened on the eggshell, exposing the CAM. The window was covered with cellophane tape, and the eggs were returned to the incubator until day 9, when the test compounds were applied. Cidofovir was placed on sterile plastic discs (Ø 8 mm), which were allowed to dry under sterile conditions. A solution of cortisone acetate (100 μg/disc; Sigma Chemical Co., St. Louis, MO) was incorporated in all discs to prevent an inflammatory response. The loaded and dried control discs (containing PBS) were placed on the CAM 1 cm away from the disc containing cidofovir. Next, the windows were covered, and the eggs were incubated until day 11, when angiogenesis was assessed. At day 11, the eggs were flooded with 10% buffered formalin (Janssen Chimica, Geel, Belgium), the plastic discs were removed, and the eggs were kept at room temperature for at least 2 h. A large area around the discs was cutoff and placed on a glass slide, and the vascular density index was measured by the method of Harris-Hooker *et al.* (39). Briefly, a grid containing three concentric circles of 4-, 5-, and 6-mm diameter was positioned on the surface of the CAM previously covered by the disc. Next, all vessels intersecting the circles were counted. The two-tailed paired Student's *t* test was used to assess the significance of the obtained results.

Chick Embryo Allantoic Sac Assay. Fertilized eggs were incubated for 3 days at 37°C, when 3 ml of albumen were removed and a window was opened on the eggshell exposing the CAM. The window was covered with cellophane tape, and the eggs were returned to the incubator. At day 8, 200 μl of PBS containing 200,000 FGF2-*T*-MAE cells were injected into the allantoic sac. Cidofovir was injected into the allantoic sac at the same time. At day 12, a large chorioallantoic vein of the CAM was perfused with India ink. Next, the CAMs were fixed, sectioned, rehydrated, and examined microscopically.

Tumor Growth in Mice. Eight-week-old female SCID mice, weighing about 20 g, were inoculated i.p. with 200 μl of MEM containing 5 × 10⁵ FGF2-*T*-MAE cells. SCID mice, weighing ~15 g, were inoculated intracerebrally into the right cerebral hemisphere with 50 μl of MEM containing 10⁵ FGF2-*T*-MAE cells. Cidofovir therapy was started 1 day after inoculation of the cells, according to a variety of treatment and administration schedules, as indicated in "Results." Mortality was recorded daily, and statistical significance of the data was assessed using the two-tailed unpaired Student's *t* test.

Eight-week-old female, athymic, nude *nu/nu* mice, weighing ~25 g, were inoculated s.c. with 100 μl of MEM containing 10⁶ FGF2-*T*-MAE cells. i.t. administration of cidofovir or MEM was started 11 days after tumor cell inoculation, *i.e.*, when tumors reached a volume of ~100 mm³. Tumor size was monitored three times a week by means of a caliper, and the tumor volume was calculated with the following formula: Tumor volume (mm³) = 0.5 × *a* × *b*², where *a* is the longest diameter and *b* is the shortest diameter.

Histological Analysis and Immunohistochemistry of Vascular Tumors. Nontreated and cidofovir-treated mice were dissected at different times after tumor cell inoculation. Tumors were fixed in 10% buffered formaldehyde and embedded in paraffin, and deparaffinized sections were subsequently stained with H&E and examined microscopically.

For immunohistochemistry, deparaffinized sections of the tumors were

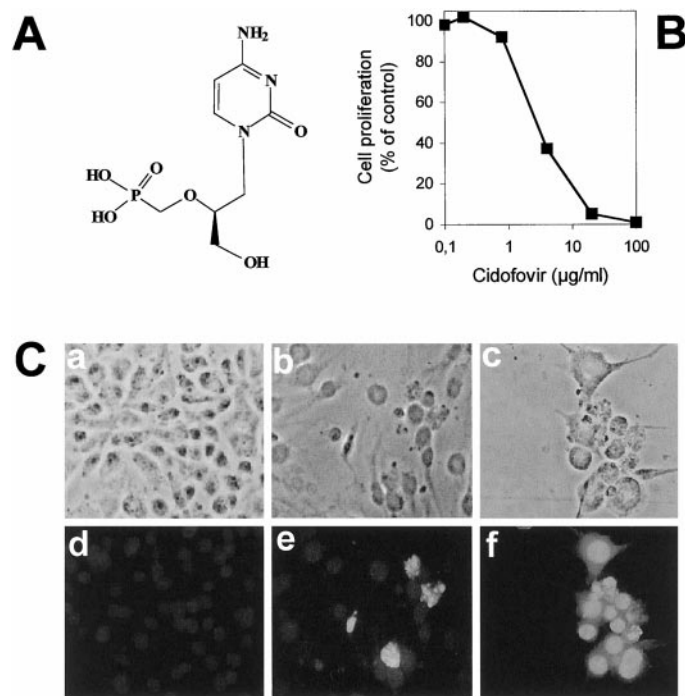


Fig. 1. Effect of cidofovir on FGF2-*T*-MAE cells. A, chemical structure of cidofovir. B, effect on cell proliferation. Cells were seeded at 20,000 cells/cm² in DMEM with 10% FCS. After 24 h, the medium was replaced, and cidofovir was added. The cell cultures were incubated for 6 days, trypsinized, and counted. C, induction of apoptosis. Subconfluent cell cultures were treated with cidofovir for 1–4 days. Next, cells were fixed, and apoptosis was determined by TUNEL staining. Apoptotic cells were visible after 3 days of treatment with 50 μg/ml (b and e) or 250 μg/ml (c and f) of cidofovir. Few apoptotic cells were detected in untreated control cultures (a and d). Phase contrast pictures are shown on the upper panel (a–c), and corresponding areas that display fluorescein-labeled DNA strand breaks after TUNEL staining are presented in the lower panel (d–f).

Table 1 Effect of cidofovir on hemangioma induction by FGF2-T-MAE cells on the CAM

FGF2-T-MAE cells ($2 \times 10^5/200 \mu\text{l}$) were inoculated together with cidofovir into the allantoic sac of chick embryos at day 8. At day 12, a large chorioallantoic vein of the CAM was perfused with India ink. Next, the CAMs were fixed, sectioned, rehydrated, and examined microscopically.

Treatment	No. of embryos		
	Treated	Dead	With hemangiomas
FGF2-T-MAE cells + 0 μg cidofovir	10	3	7
FGF2-T-MAE cells + 1 μg cidofovir	10	2	8
FGF2-T-MAE cells + 10 μg cidofovir	10	3	6
FGF2-T-MAE cells + 100 μg cidofovir	10	3	0

incubated for 1 h with 1.5% blocking serum in PBS. After removal of the blocking serum, the slides were incubated with a primary mouse monoclonal antibody directed against human PCNA (clone PC10; Calbiochem-Novabiochem, Nottingham, United Kingdom) for 30 min at room temperature. The slides were subsequently washed in PBS for 5 min and then incubated with biotinylated secondary antimouse antibody (Dako, Glostrup, Denmark) for 30 min at room temperature. After a 5-min wash, all slides were incubated with horseradish peroxidase-labeled streptavidin (Dako Corp.) for 30 min at room temperature. The immunoreactions were visualized as brown precipitates by incubation with the substrate diaminobenzidine (Dako) for 5 min. The reaction was stopped by water, and the slides were counterstained with hematoxylin. Apoptosis detection in the tumors was performed by means of the *In Situ* Cell Death Detection kit (Boehringer Mannheim), as described above. After TUNEL staining, tissue sections were washed in PBS and exposed to the DNA-binding dye HOECHST 33342 (Sigma Chemical Co.) at a concentration of 1.2 $\mu\text{g}/\text{ml}$ in PBS for 15 min at room temperature. The slides were extensively washed in PBS and examined with a Zeiss fluorescence microscope. Apoptotic cells were identified by positive TUNEL staining and chromatin condensation.

RESULTS

Effect of Cidofovir on FGF2-T-MAE Cell Growth and Apoptosis *in Vitro*. We reported recently on the potent inhibition of PyV-induced hemangioma development in rats by cidofovir. This activity was not mediated by an antiviral effect (29). To elucidate the mechanism by which cidofovir abrogates vascular tumor growth, we used FGF2-T-MAE cells. These endothelial cells are not transformed by an oncogenic virus (as is the case for PyV-induced hemangiomas) but overexpress FGF2 and induce vascular tumors on the CAM and in nude mice (35, 36).

In vitro, cidofovir caused a dose-dependent inhibition of FGF2-T-MAE cell proliferation with a CC_{50} of 6.7 $\mu\text{g}/\text{ml}$ after 6 days of treatment (Fig. 1B). To study the reversibility of the observed growth-inhibitory effect on FGF2-T-MAE cells, cidofovir was removed after 48 h, and cultures were kept for 4 days in the absence of the compound. At 10 $\mu\text{g}/\text{ml}$, removal of cidofovir resulted in accelerated cell proliferation. In contrast, the cells did not recover if exposed to cidofovir at 30 $\mu\text{g}/\text{ml}$, indicating that this concentration is toxic rather than cytostatic (data not shown).

Next, subconfluent FGF2-T-MAE cells, grown in four-well chamber slides, were incubated with various concentrations of cidofovir for 1, 2, 3, or 4 days, after which induction of apoptosis was assessed. No apoptotic cells were identified at cidofovir concentrations $<25 \mu\text{g}/\text{ml}$. At 25 $\mu\text{g}/\text{ml}$, few apoptotic cells were visible after 3 days of cidofovir treatment. At higher concentrations (50–100–250 $\mu\text{g}/\text{ml}$), apoptosis was evident after exposure of the cells to the compound for 48 h. No viable cells were detected after 3 days at 250 $\mu\text{g}/\text{ml}$ (Fig. 1C). The majority of these cells showed membrane blebbing and the presence of apoptotic bodies, hallmarks of apoptotic cell death.

Effect of Cidofovir on Hemangioma Growth and Angiogenesis on the CAM. Upon inoculation into the allantoic sac of chick embryos, FGF2-T-MAE cells induce the formation of hemangiomas on

the CAM as well as an increase in overall CAM angiogenesis (36, 37). When FGF2-T-MAE cells were injected alone, hemangiomas developed in all embryos (Table 1; Fig. 2A). However, no hemangiomas were observed when the cells were coinoculated in the allantoic sac with 100 μg of cidofovir; lower concentrations of the drug were ineffective. Interestingly, cidofovir did not affect the vascularization of the CAM at all doses tested (not shown), suggesting that cidofovir is not endowed with a potent antiangiogenic activity. Indeed, only a moderate antiangiogenic effect was noted when cidofovir was applied topically on the CAM; maximal inhibition ($34.7\% \pm 11.9$; $P < 0.05$) was achieved with 200 μg of cidofovir (Fig. 2B).

Effect of Cidofovir on the s.c. Growth of FGF2-T-MAE Tumors in Nude Mice. We next evaluated the effect of cidofovir on FGF2-T-MAE cell-induced tumors in immunodeficient mice. s.c. inoculation of 10^6 FGF2-T-MAE cells in nude mice resulted in the appearance of tumor lesions at day 6. These tumors have been classified previously as hemangioendotheliomas, consisting of spindle-shaped cells resembling KS and numerous CD31-positive blood vessels and lacunae (35). Eleven days after cell inoculation, when tumors had reached a volume of $\sim 100 \text{ mm}^3$, intratumoral treatment with cidofovir was initiated. Daily administration of cidofovir at 25 mg/kg for 2 weeks completely abolished tumor growth and resulted in tumor disappearance in 38% of the animals at day 25. These mice remained tumor free for the next month (Fig. 3). When treatment was terminated, remain-

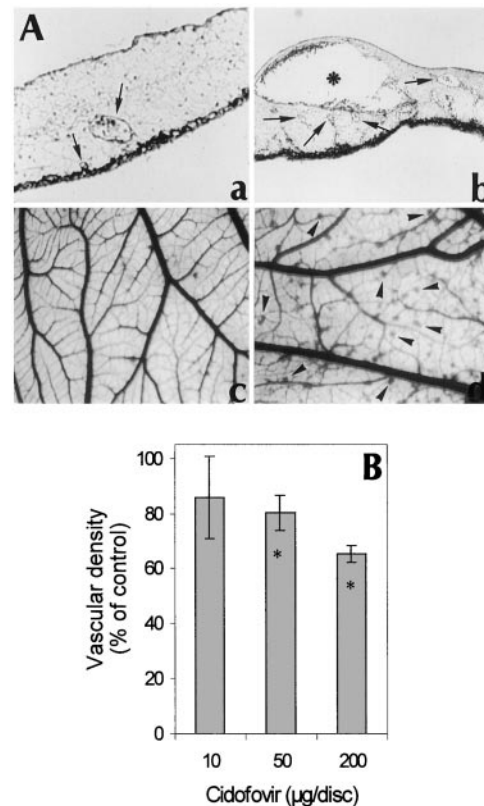


Fig. 2. Effect of cidofovir on hemangioma formation and angiogenesis in the CAM. A, FGF2-T-MAE cells were inoculated into the allantoic sac of chick embryos at day 8, without (b and d) or together with 100 μg of cidofovir (a and c). The CAM vasculature was visualized by i.v. injection of India ink at day 12 (c and d), followed by histological analysis (a and b). Numerous hemangiomas of various sizes were induced by FGF2-T-MAE cells (b, $\times 160$; d, $\times 10$), whereas addition of 100 μg of cidofovir completely abolished hemangioma formation on the CAM (a, $\times 160$; c, $\times 10$). Normal blood vessels (arrows) and hemangiomas (asterisk or arrowheads) are indicated. B, discs containing either cidofovir or PBS were applied topically on the CAM on day 9. At day 11, the number of blood vessels under the discs was determined. Results are expressed as the mean percentage of control ($n = 10$ –25) and are compared by paired Student's *t* test. *, statistical difference between control and test; $P < 0.05$.

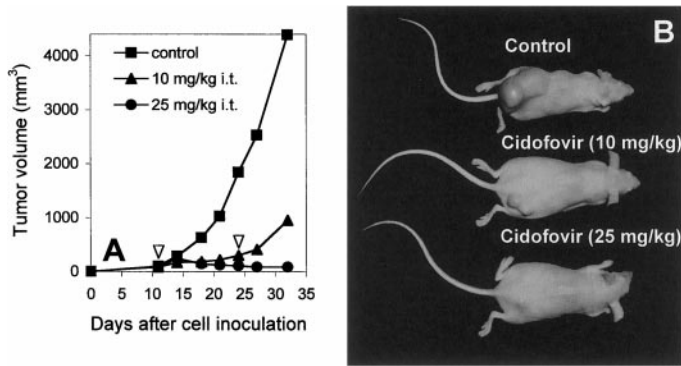


Fig. 3. Effect of cidofovir on the s.c. growth of FGF2-T-MAE xenografts in nude mice. Nude mice were inoculated s.c. with 10^6 FGF2-T-MAE cells. Animals were treated i.t. daily with 25 or 10 mg/kg of cidofovir from day 11 till day 24 (arrowheads). Tumor size was monitored three times a week, and the tumor volume was calculated with the following formula: Tumor volume (mm^3) = $0.5 \times a \times b^2$, where a is the longest diameter and b is the shortest diameter (A). Cidofovir therapy caused stabilization (10 mg/kg) or regression (25 mg/kg) of the tumors (B).

ing tumors increased in size, although at a much slower rate than in the untreated animals. Under the same experimental conditions, cidofovir also elicited a marked inhibition of tumor growth when administered at 10 mg/kg/day (Fig. 3).

Histological analysis showed necrotic areas in all cidofovir-treated tumors ($n = 6$) already after 2 days of treatment. At this time point, necrosis was absent in tumors of untreated control mice ($n = 6$; Fig. 4, A and B). Immunohistochemical staining of the tumor sections by PCNA showed extensive proliferation of tumor cells in both untreated and cidofovir-treated mice, except in the necrotic tumor areas (Fig. 4, C–F). Cidofovir induced apoptosis at 2 days after the beginning of treatment, as evidenced by TUNEL staining of tumor sections (Fig. 4J) and subsequent counterstaining with Hoechst reagent (Fig. 4H). Cells that stained positive with the TUNEL technique also showed nuclear condensation, which is characteristic for cell death by apoptosis. In contrast, tumor tissues from control animals were TUNEL negative (Fig. 4I) and displayed a normal DNA staining pattern (Fig. 4G).

Effect of Cidofovir on Survival of SCID Mice Bearing i.p. FGF2-T-MAE Xenografts. After i.p. inoculation in SCID mice, FGF2-T-MAE cells developed tumors macroscopically visible at autopsy 12 days after cell inoculation. Tumor growth was associated with anemia, leading to death of the animals within 4 weeks after cell inoculation (Fig. 5). In a first set of experiments, the activity of cidofovir was compared with that of the two other nucleoside analogues: ganciclovir, an antiviral agent with comparable *in vitro* cytostatic activity as cidofovir (data not shown); and cytarabine (ara-C), a chemotherapeutic agent with established antitumoral activity. All compounds were administered i.p., cidofovir at 50 mg/kg, 3 times a week and ganciclovir and ara-C at the highest tolerable dose, which corresponds to 50 mg/kg, 5 times a week for both compounds. Treatment was initiated 1 day after cell inoculation and was continued until the first placebo-treated animal died (MDD, 27.4 ± 2.8). Ganciclovir did not inhibit tumor growth (MDD, 30.3 ± 3.9), whereas ara-C and cidofovir significantly prolonged the life span of the animals [MDD, 49.5 ± 8.4 ($P < 0.001$) and 40.3 ± 3.9 ($P < 0.001$) for the cidofovir- and ara-C-treated groups, respectively]. The antitumoral activity of cidofovir was thus more pronounced than that of ara-C ($P < 0.05$), although cidofovir was administered at a total weekly dose of 150 mg/kg versus 250 mg/kg for ara-C (Fig. 5).

Cidofovir also proved highly effective after infrequent dosing. When administered at 150 mg/kg, once a week, for 4 consecutive weeks, the mean life span of the mice increased from 26.4 ± 0.9 days

for the untreated control group to 55.0 ± 7.9 days ($P < 0.001$) for the cidofovir-treated animals (Fig. 6A). At 3 weeks, when untreated mice had developed large tumors in the peritoneal cavity (Fig. 6B), cidofovir-treated mice did not show any macroscopic sign of tumor development (Fig. 6C). In a parallel group, treatment was continued for 8 consecutive weeks. During this treatment period, small tumors started to develop after 7 weeks and led to death of the animals at 74.6 ± 7.4 days ($P < 0.001$; Fig. 6A) after cell inoculation. Histological examination did not reveal any metastases in lungs, liver, spleen, or kidney from either control or treated groups. Also, prolonged treatment with cidofovir did not cause toxicity in any of these organs.

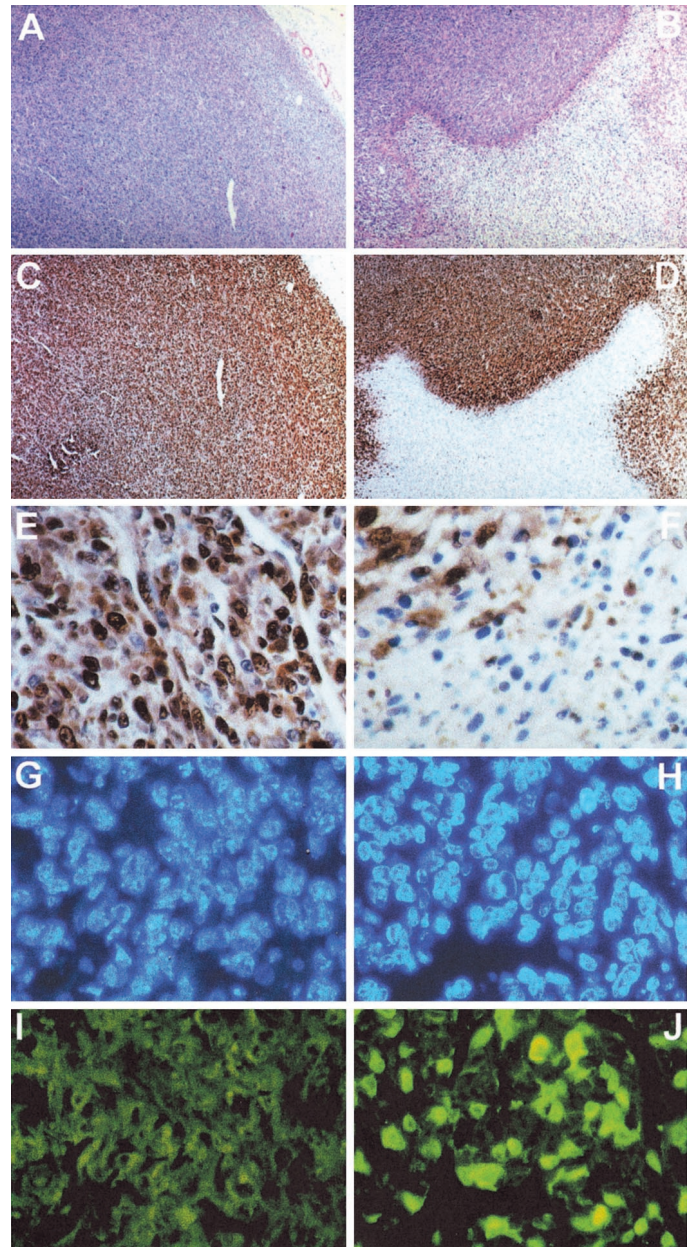


Fig. 4. Histological appearance of FGF2-T-MAE tumors treated with cidofovir. Nude mice were inoculated s.c. with 10^6 FGF2-T-MAE cells. At days 11 and 12 after cell inoculation, animals were treated i.t. with 25 mg/kg of cidofovir. At day 13, 3F2T tumors were dissected and fixed. Sections of tumors, either untreated ($n = 6$; A, C, E, G, and I) or treated for 2 days with cidofovir ($n = 6$; B, D, F, H, and J) were stained with H&E (A and B), PCNA (C–F), Hoechst 33342 (G and H), or TUNEL (I and J). A–D, $\times 125$; E–J, $\times 200$. In D and F, PCNA-negative regions represent areas of necrosis. The weak fluorescence in I is attributable to background signal.

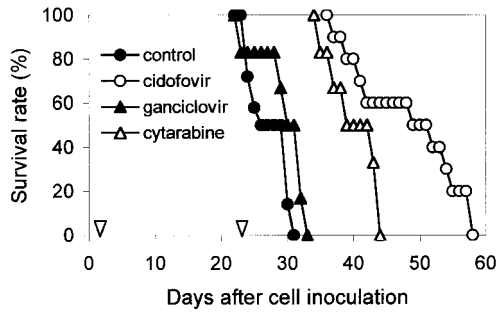


Fig. 5. Effect of nucleoside analogues on animal mortality caused by i.p. FGF2-T-MAE tumors. FGF2-T-MAE cells (5×10^5) were inoculated i.p. in SCID mice at day 0. Ganciclovir and ara-C were administered i.p., at 50 mg/kg, five times per week. Cidofovir was injected i.p., at 50 mg/kg, three times per week. Treatment was started at day 1 and continued until the first untreated animal had died (arrowheads).

In the next set of experiments, cidofovir was administered s.c., i.e., at a distant site from the growing tumors. When given at 100 mg/kg, three times a week, from day 1 until death of the first untreated mouse, cidofovir caused a significant delay in tumor-associated mortality [MDD, 44.9 ± 7.0 ($P < 0.001$) versus 28.8 ± 2.4 for the control group]. Even when given s.c., once weekly, for 3 consecutive weeks at 100 mg/kg, cidofovir still caused a 10-day delay in tumor-associated mortality [MDD, 38.2 ± 1.6 ($P < 0.001$); not shown].

Effect of Cidofovir on FGF2-T-MAE Tumors in the Brain.

Finally, the activity of systemically administered cidofovir was evaluated against the development of brain FGF2-T-MAE tumor lesions. Tumor cell infiltration and the development of small intracerebral tumors could be observed 17 days after cell inoculation in the brain of SCID mice (Fig. 7B). Then, the rapid growth of the tumor (Fig. 7, C and D) led to mortality after 23.6 ± 1.9 days (Fig. 7A). i.p. injection of cidofovir at 50 mg/kg, 3 times a week, starting at day 1, significantly protected the mice against intracerebral tumor growth (Fig. 7A). Indeed, cidofovir-treated animals were still tumor free at day 23 (Fig. 7E). However, when cidofovir treatment was terminated, tumor growth resumed, although mortality was significantly delayed to 43.8 ± 6.1 days after tumor cell inoculation ($P = 0.001$).

DISCUSSION

Cidofovir is a broad-spectrum, anti-DNA virus agent with activity against herpesvirus, poxvirus, adenovirus, papillomavirus, and polyomavirus infections (6). The compound has been approved for the treatment of cytomegalovirus retinitis in AIDS patients. However, cidofovir has also shown clinical efficacy in the treatment of (muco)cutaneous HSV-1 and HSV-2 infections (7-9), adenovirus conjunctivitis (40), poxvirus infections (21, 22), JC

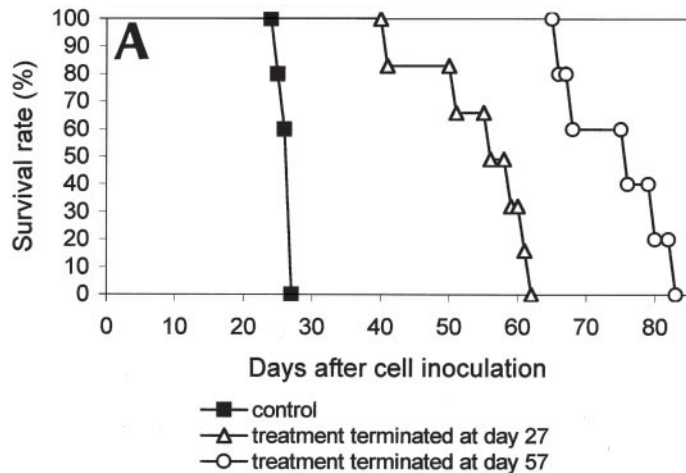
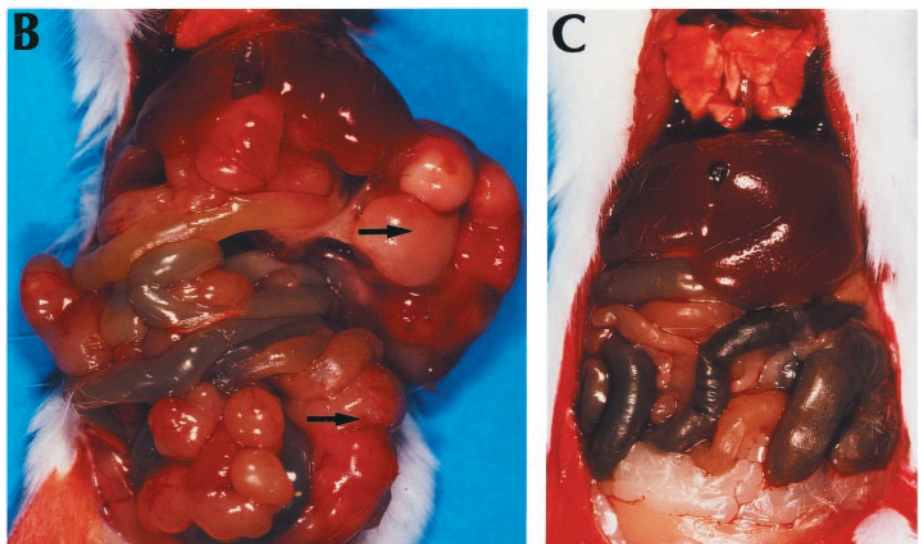


Fig. 6. Effect of cidofovir on growth of i.p. FGF2-T-MAE tumors and animal mortality. FGF2-T-MAE cells (5×10^5) were inoculated i.p. in SCID mice at day 0. Cidofovir was administered i.p., at 150 mg/kg, once weekly. Treatment was initiated at day 1 and continued for 4 weeks (until death of the control animals) or 8 weeks (until death of the 4-week treatment group) (A). At regular time points, animals were dissected and examined histologically. Three weeks after cell inoculation, control mice had large tumors (B, arrows), which were highly proliferative. Macroscopic tumors were not detectable in the treated mice after 3 weeks (C).



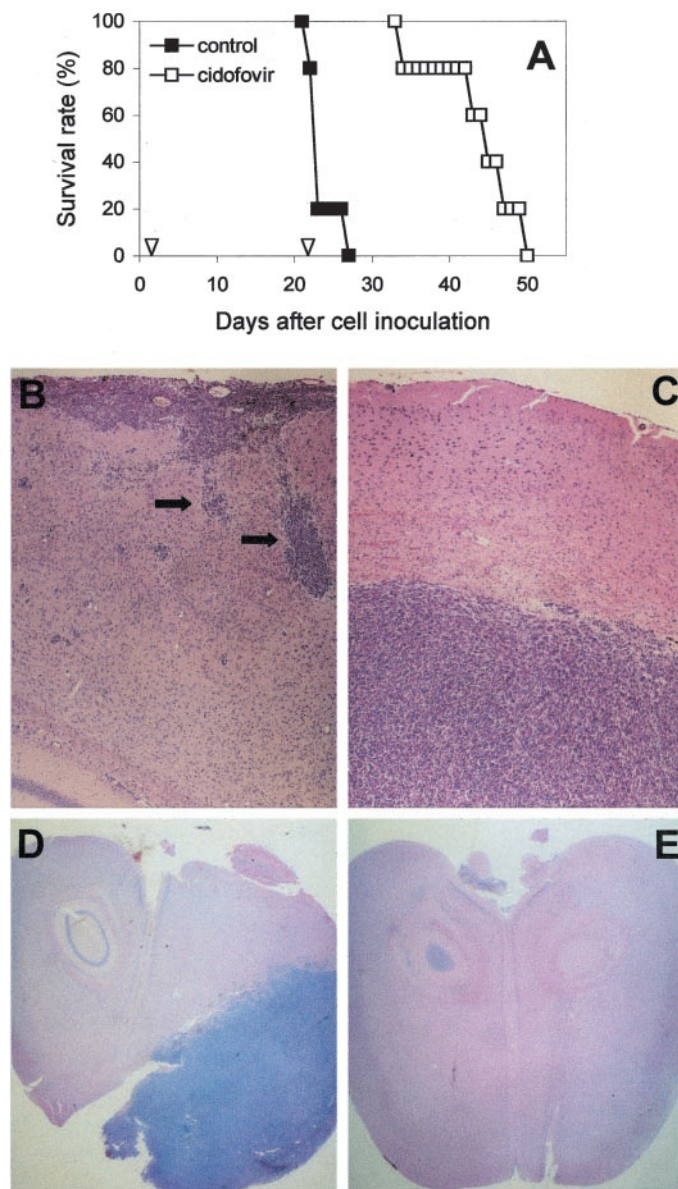


Fig. 7. Effect of cidofovir on mortality associated with the growth of intracerebral FGF2-T-MAE tumors. FGF2-T-MAE cells (10^5) were inoculated intracerebrally in SCID mice. Cidofovir was administered i.p., at 50 mg/kg, three times per week, starting at day 1 after inoculation and continued for 3 consecutive weeks (until death of the control mice; A, arrowheads). Histological section of the brain at 17 days after tumor cell inoculation showed a small tumor infiltrating the brain from the site of cell inoculation (B, arrows). At day 23 after inoculation, the tumor had destroyed large parts of the brain (C and D). At the same time point, the brains of cidofovir-treated animals were still tumor free (E). B and C, $\times 200$; D and E, $\times 50$.

virus-induced progressive multifocal leukoencephalopathy (10, 11), and EBV-induced oral hairy leukoplakia (41).

In addition to its antiviral activity, a potent antitumor activity has been attributed to cidofovir. When applied topically or intratumorally, the compound caused a marked to complete regression of HPV-associated lesions in patients (18–20). Furthermore, we have demonstrated that cidofovir is highly effective against EBV-transformed NPC tumors (27) and PyV-induced hemangiomas in animals (29). Thus, cidofovir appears to exert a potent antitumor activity against oncogenic virus-induced lesions. Viruses such as EBV, HPV, and PyV encode for cell-transforming proteins, which have been shown to interact with products of tumor suppressor genes (42–45), raising the possibility that cidofovir may exert its antitumor action by interfering

with this interaction, thus restricting its activity to virus-transformed tumors.

In this study, we have demonstrated that cidofovir inhibits the growth of strongly vascularized tumors, previously characterized as hemangioendotheliomas (35, 36), induced by inoculation of FGF2-overexpressing FGF2-T-MAE cells in immunodeficient mice. This is the first demonstration of the antitumor activity of cidofovir against tumor cells not associated with an oncogenic virus. Intratumoral therapy with cidofovir resulted in complete regression of s.c. lesions. Furthermore, an important antitumoral effect of cidofovir was observed also when the compound was injected at a site distant from the xenograft, *i.e.*, s.c. versus i.p. growing tumors and i.p. versus intracerebrally growing tumors. The antitumoral activity of cidofovir was even more pronounced than that of ara-C, akin to cidofovir, a cytosine-based nucleoside analogue. Cidofovir proved also very effective when administered infrequently, *i.e.*, once a week. This may partly be explained by the long intracellular half-life of the HPMPCp-choline metabolite (15) and corroborates the long-lasting antiviral activity of this molecule, as observed in cell cultures, animal models, and the clinical setting. Moreover, ganciclovir, a structurally related nucleoside analogue with a comparable *in vitro* cytostatic activity as cidofovir (data not shown), did not elicit any antitumor activity. This indicates that the *in vivo* activity of cidofovir cannot be explained solely by its inhibitory effect on FGF2-T-MAE cell proliferation.

Tumor growth is determined by the balance between cell proliferation and apoptosis and can be modulated by angiogenesis (46). The potent systemic activity of cidofovir against FGF2-transformed vascular tumors may suggest that the molecule is endowed with antiangiogenic properties. However, although cidofovir inhibits the proliferation of primary (data not shown) and transformed endothelial cells, the compound did not influence the capacity of FGF2-T-MAE cells (36, 37) to sprout in three-dimensional fibrin gel and to undergo morphogenesis on Matrigel (data not shown). Also, cidofovir completely abrogated hemangioma development induced by FGF2-T-MAE cells injected in the allantoic sac of chick embryos without affecting the growth of the normal CAM vessels. Moreover, local application of cidofovir caused a limited inhibition of neovascularization in the “conventional” CAM assay. Taken together, the data indicate that the primary action of this drug is not angiostatic. Inhibition of tumor vascularization may, however, be a secondary effect, resulting from the antiproliferative activity of this drug against different cell types, including endothelial cells. Relevant to this point, it has recently been postulated that chemotherapeutic drugs, which are primarily used at high doses to kill the cancer cells, can also be antiangiogenic. Because these drugs are not specific, they may also inhibit the proliferation of other cell types, including intratumoral endothelial cells, which are highly proliferative. Therefore, long-term, regular administration of chemotherapeutics at low doses might increase their antitumoral potential. Indeed, antiendothelial and antiangiogenic effects have been demonstrated for several cytotoxic compounds (47–49). Moreover, an alternative antiangiogenic schedule for administration of the anticancer agent cyclophosphamide increased apoptosis of: (a) the endothelial cells within the tumor vasculature; and (b) cyclophosphamide-resistant tumor cells, resulting in significant improvement over the conventional schedule and eradication of the tumors (49).

Cidofovir caused a time- and dose-dependent induction of apoptosis in FGF2-T-MAE cells *in vitro*. At concentrations $<25 \mu\text{g/ml}$, the drug was cytostatic, whereas higher concentrations led to cell death induced by apoptosis. We next characterized the effects of cidofovir treatment on proliferation and apoptosis in FGF2-T-MAE tumors. After 2 days of treatment, a marked increase in the number of apoptotic cells was noted in the cidofovir-treated xenografts, whereas

tumor cell proliferation was unaffected. These data are in agreement with previous observations on NPC xenografts in nude mice in which cidofovir caused extensive apoptosis leading to tumor regression (27).

A number of antitumor agents, including the nucleoside analogues cytarabine (ara-C), gemcitabine, and 5-fluorouracil, elicit their antitumor activity by induction of apoptosis (50, 51). Compared with these drugs, cidofovir possesses low *in vitro* cytostatic and cytotoxic action. For instance, cidofovir is ~500-fold less toxic than ara-C that induced apoptosis of FGF2-*T*-MAE cells *in vitro* at 0.5 µg/ml (data not shown). The signaling pathway leading to drug-induced apoptosis has remained largely unknown and does not seem to be mediated by FAS receptor activation (52). The capacity of cidofovir to induce apoptosis in p53 deletion mutant NPC tumors rules out the involvement of p53 in mediating the antitumor activity of this drug (27). Gemcitabine monophosphate results in apoptosis because of incorporation into cellular DNA (53). Interestingly, cidofovir also possesses the potential to become integrated into DNA (17). Alternatively, cidofovir might disrupt the cell cycle, which has been shown to trigger apoptosis after 5-fluorouracil treatment (54). Recent experiments have demonstrated a marked increase in CPP32 (caspase-3) protease activity caused by cidofovir in HPV-containing cells (55).

FGF2 is an important mediator in the progression of KS (33). In apparent contrast with its potent antitumoral activity against FGF2-overexpressing endothelial cells, intratumorally administered cidofovir did not afford a protective effect on KS lesion progression in mice⁴ as well as in the clinical setting (23). This indicates significant molecular differences between the FGF2-*T*-MAE cell-induced hemangioendotheliomas and KS lesions. Recent data indicate that chemotherapeutic resistance of KS cells could be attributed to the long doubling times of these cells (56). Indeed, stimulation of KS cells with FGF2 rendered them more sensitive to the cytostatic activity of cidofovir, as indicated by the markedly reduced CC₅₀ of cidofovir after FGF2 stimulation (56).

In conclusion, the data presented here indicate that cidofovir should also be explored for the treatment of vascular tumors (that are not associated with an oncogenic virus), such as juvenile hemangiomas. The ability to cross the blood-brain barrier and to suppress the progression of angiogenic brain tumors after systemic treatment warrants further investigation for the use of cidofovir in the therapy of gliomas, highly proliferative vascular brain lesions for which at present no adequate treatment is available. Cidofovir appears to inhibit tumor growth directly by induction of cell death (apoptosis) and indirectly by inhibiting the proliferation of the endothelial cells lining the tumor vasculature. The trigger and subsequent signaling pathway leading to apoptosis activation by cidofovir is currently under investigation.

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