Acycloguanosyl 5′-thymidyltriphosphate, a Thymidine Analogue Prodrug Activated by Telomerase, Reduces Pancreatic Tumor Growth in Mice

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BACKGROUND & AIMS: Gemcitabine is the standard of care for metastatic and nonresectable pancreatic tumors. Phase II and III trials have not demonstrated efficacy of recently developed reagents, compared with gemcitabine alone; new chemotherapic agents are needed. Ninety percent of pancreatic tumors have telomerase activity, and expression correlates with tumor stage. We developed a thymidine analogue prodrug, acycloguanosyl 5′-thymidyltriphosphate (ACV-TP-T), that is metabolized by telomerase and releases the active form of acyclovir. We investigated the antitumor efficacy of ACV-TP-T in vitro and in vivo. **METHODS:** We evaluated proliferation and apoptosis of human pancreatic cancer cells (PANC-1, MiaPaca2, BxPc3, PL45, and Su.86.86) incubated with ACV-TP-T. The presence of ACV-TP-T and its metabolite inside the cells were analyzed by mass spectrometry. In vivo efficacy was evaluated in nude mice carrying PANC-1 or MiaPaca2 pancreatic xenograft tumors. **RESULTS:** The prodrug of ACV-TP-T was actively metabolized inside pancreatic cancer cells into the activated form of acyclovir; proliferation was reduced, apoptosis was increased, and the cell cycle was altered in pancreatic cancer incubated with ACV-TP-T, compared with controls. Administration of ACV-TP-T to mice reduced growth, increased apoptosis, and reduced proliferation and vascularization of pancreatic xenograft tumors. **CONCLUSIONS: ACV-TP-T, a thymidine analogue that is metabolized by telomerase and releases the active form of acyclovir, reduces proliferation and induces apoptosis of human pancreatic cancer cell lines in vitro and pancreatic xenograft tumors in mice.**

Keywords: Suicide Gene Therapy; Human Telomerase Reverse Transcriptase; hTERT; Telomere.

Pancreatic cancer continues to be a leading cause of cancer death in the Western world and its management remains the most challenging task in oncology. Although it represents only 2%–3% of all cancers, pancreatic adenocarcinoma is one of the most aggressive and fatal human malignancies, accounting for 6% of all cancer deaths.[1] The prognosis is generally poor, with a median survival of 6 months and an overall 5 years survival rate estimated at less than 5%.[1] In the last few years, many efforts have been devoted to unveil the causes of pancreatic cancer without any substantial improvement in the clinical prognosis.

Gemcitabine, a deoxycytidine nucleoside analogue and cell cycle specific inhibitor of DNA synthesis, remains the only standard of care for this disease with palliative effects.[2] Except for Erlotinib, which has demonstrated modest survival benefit in combination with gemcitabine,[3] no blocking agents for the epidermal growth factor, vascular endothelial growth factor and RAS pathways, alone or in combination, have been shown to be superior to gemcitabine.[4–6] Therefore, the development of new therapeutic interventions for this devastating disease is urgently needed, given the fact that the antitumoral therapies in place suffer from lacking target selectivity and, in most cases, cause severe adverse effects and overall systemic toxicity.[7,8]

Among the new therapeutic approaches developed for cancer therapy, much expectation has been raised by the introduction of the suicide gene therapy with adenovirus carrying the herpes virus thymidine kinase gene (TK) and acyclovir (ACV) derivatives.[9,10] ACV is a nucleoside analogue acting as a DNA chain terminator mainly applied

Abbreviations used in this paper: ACV, acyclovir; ACV-TP-dA, acycloguanosyl 2′-deoxy-5′-adenosyltriphosphate; ACV-TP-dC, acycloguanosyl 5′-cythydyltriphosphate; ACV-TP-dG, acycloguanosyl 2′-deoxy-5′-guanosyltriphosphate; ACV-TP-T, acycloguanosyl 5′-thymidyltriphosphate; ALT, alternative lengthening of telomeres; BrdU, bromodeoxyuridine; DN-hTERT, dominant negative human telomerase reverse transcriptase; GCV, ganciclovir; hTERT, human telomerase reverse transcriptase; IC_{50}, half maximal inhibitory concentration; IHC, immunohistochemistry; PCNA, proliferating cell nuclear antigen; T/C, treated/control mean tumor volume ratio; siRNA, small interfering RNA; TK, thymidine kinase gene.

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in the treatment of herpes virus infections.[9] Inside infected cells, ACV undergoes 3 consecutive phosphorylations yielding the active metabolite (ACV triphosphate); the first phosphorylation is performed only by viral TK and the remaining 2 by cellular kinases. The active metabolite is then incorporated into DNA during its replication causing DNA chain termination. In the suicide gene therapy, ACV or the ACV analogue ganciclovir (GCV) are activated by TK carried in the recombinant viral genome.[9,10] This approach has been extensively applied in in vitro studies against various types of tumors including pancreatic cancer[11,12]; however, it raises ethical concerns regarding the virus utilization in patients[13,14] and is undermined by the risk of the low and transient expression levels of the transgene.[9,13,14]

Studies on nucleotide mimics, created as new drugs for the treatment of human immunodeficiency virus infection and characterized by a bulky group substitution in the γ-position, demonstrated that these molecules are preferentially substrates of human immunodeficiency virus reverse transcriptase but are poorly utilized by the host DNA polymerase α.[15,16] In replicating cells, a reverse-transcriptase ribonucleoprotein, deputed to maintain telomeres integrity, is telomerase.[17] In normal conditions, this enzyme is repressed in the majority of adult somatic cells, but it is reactivated during tumorigenesis[18] and is considered to be involved in cell immortality and cancer growth.[19] Interestingly, 90% of pancreatic tumors show telomerase activity and telomerase expression correlates with the tumor staging.[20]

These evidences prompted us to realize an ACV-derived prodrug that could be selectively metabolized inside cancer cells in the active form by telomerase without exploiting the viral TK activity. This synthetic prodrug, acycloguanosyl 5′-thymidyltriphosphate (ACV-TP-T), is constituted by a thymidine triphosphate attached by the γ-phosphate to the hydroxyl group of ACV, and it should be used by telomerase to incorporate the thymidine in the replicating telomeres directly releasing ACV diphosphate that is transformed in the active triphosphate form by cellular kinases (Figure 1 and Supplementary Figure 1). In the present paper, we report data demonstrating ACV-TP-T mode of action and the testing of its activity in vitro and in vivo against pancreatic tumors, with very encouraging results.

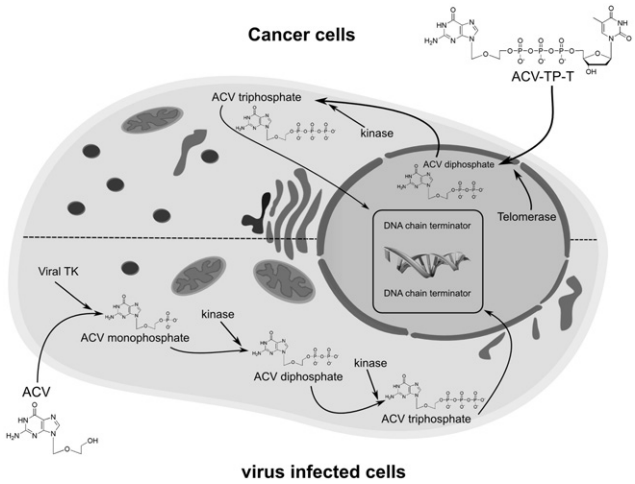

Figure 1. Structure and schematic mode of action of ACV-TP-T in comparison with ACV. For activation, ACV requires to be phosphorylated to ACV monophosphate by viral TK carried either by wild-type herpes virus or, in the suicide gene therapy, engineered adenovirus (*lower part of the Figure*). ACV monophosphate is then further phosphorylated by cellular kinases to the triphosphated active form. Conversely, ACV-TP-T is a substrate of telomerase that incorporates the thymidine in the replicating telomeres and directly releases ACV diphosphate skipping the viral TK phosphorylation step (*upper part of the Figure*).

Materials and Methods

Cell Culture

Human pancreatic adenocarcinoma cell lines (PANC-1, MiaPaca2, BxPc3, PL45, and Su.86.86) were purchased from the American Type Culture Collection (Manassas, VA). Stock cell lines were routinely cultured in Dulbecco's modified Eagle medium (PANC-1, MiaPaca2, BxPC3, PL45), or Iscove's (Su.86.86) supplemented with 10% fetal bovine serum, L-glutamine, and antibiotics (50 U/mL penicillin, 50 μg/mL streptomicin).

Telomerase Activity Assay

Telomerase activity was tested by the TeloTAGGG Telomerase PCR ELISA PLUS kit (Roche, Milan, Italy). Briefly, 2×10^5 exponentially growing cells were lysated using the provided lysis buffer then the assay was run according to manufacturer protocol.

Detection of Prodrug Metabolites

PANC-1 cells (5×10^6 cells) were incubated with 1 mmol/L final concentration of ACV-TP-T. After 2 hours, cells were lysated with ice-cold 60% methanol-40% 15 mmol/L ammonium acetate buffer (pH 6.7). Cellular lysates were then transferred in YM-3 3000 MW cut-off Centricon tubes (Millipore, Milan, Italy) and centrifuged; the filtrated solutions were used for liquid chromatography tandem mass spectrometry analysis. The analysis was performed with LTQ linear ion trap mass spectrometer (Thermo's Finnigan, Milan, Italy) with the combined workflow of nano liquid chromatography tandem mass spectrometry (electrospray mode). The liquid chromatography separation was performed using a Zic-Hilic 150 × 2.1 mm (Sequant, Umea, Sweden) eluted at a flow rate of 250 μL/min. The mobile phase A was a 20-mmol/L ammonium acetate solution (pH 7.5), whereas solvent B was acetonitrile. The gradient applied was from 85% to 50% of B in 18 minutes. The concentration of ACV-TP-T in the cells was estimated by comparison of the peak area of a