Research Article

EASL EUROPEAN ASSOCIATION FOR THE STUDY OF THE LIVER | JOURNAL OF HEPATOLOGY

Telomerase activated thymidine analogue pro-drug is a new molecule targeting hepatocellular carcinoma

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Background & Aims: Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide. Although hepatectomy and transplantation have significantly improved survival, there is no effective chemotherapeutic treatment for HCC and its prognosis remains poor. Sustained activation of telomerase is essential for the growth and progression of HCC, suggesting that telomerase is a rational target for HCC therapy. Therefore, we developed a thymidine analogue pro-drug, acycloguanosyl-5′-thymidyltriphosphate (ACV-TP-T), which is specifically activated by telomerase in HCC cells and investigated its anti-tumour efficacy.

Methods: First, we verified *in vitro* whether ACV-TP-T was a telomerase substrate. Second, we evaluated proliferation and apoptosis in murine (Hepa1-6) and human (Hep3B, HuH7, HepG2) hepatic cancer cells treated with ACV-TP-T. Next, we tested the *in vivo* treatment efficacy in HBV transgenic mice that spontaneously develop hepatic tumours, and in a syngeneic orthotopic murine model where HCC cells were implanted directly in the liver.

Results: *In vitro* characterization provided direct evidence that the pro-drug was actively metabolized in liver cancer cells by telomerase to release the active form of acyclovir. Alterations in cell cycle and apoptosis were observed following *in vitro* treatment with ACV-TP-T. In the transgenic and orthotopic mouse models, treatment with ACV-TP-T reduced tumour growth, increased apoptosis, and reduced the proliferation of tumour cells.

Conclusions: ACV-TP-T is activated by telomerase in HCC cells and releases active acyclovir that reduces proliferation and induces apoptosis in human and murine liver cancer cells. This pro-drug holds a great promise for the treatment of HCC.

Cancer

Keywords: Hepatocellular carcinoma; Telomerase; Acyclovir; Cancer therapy; hTERT.

Received 22 November 2013; received in revised form 22 April 2014; accepted 11 May 2014; available online 23 May 2014

☆ DOI of original article: http://dx.doi.org/10.1016/j.jhep.2014.08.017.

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Abbreviations: ACV, acyclovir; ACV-DP, acyclovir diphosphate; ACV-TP, acyclovir triphosphate; ACV-TP-dA, acycloguanosyl 2-deoxy-5-adenosyltriphosphate; ACV-TP-C, acycloguanosyl 5-cythydyltriphosphate; ACV-TP-dG, acycloguanosyl 2-deoxy-5-guanosyltriphosphate; ACV-TP-T, acycloguanosyl 5-thymidyltriphosphate; BrdU, bromodeoxyuridine; DN-hTERT, dominant negative human telomerase reverse transcriptase; HCC, hepatocellular carcinoma; hTERT, human telomerase reverse transcriptase; IC_{50}, half maximal inhibitory concentration; IHC, immunohistochemistry; PCNA, proliferating cell nuclear antigen.

Introduction

Hepatocellular carcinoma (HCC) is the primary malignancy of hepatocytes and the most frequent solid tumour of the liver. Half a million cases occur annually in the world making it the fifth most common malignancy in men and the ninth in women [1]. Hepatocarcinogenesis is a multistep process, involving genetic and epigenetic events that accumulate during chronic liver diseases. The extent of hepatic dysfunction limits therapeutic options for HCC and the prognosis of patients with this tumour remains dismal, as the average survival from the time of diagnosis of unresectable HCC is measured in months [2]. Therefore, in this dramatic scenario, HCC is an attractive target for the identification of new chemotherapeutic agents.

The current trend in research on anti-cancer drugs is to exploit particular traits or hallmarks that are unique to cancer cells. Despite the fact that cancers display a great heterogeneity in clinical behaviour, most human tumours, including HCC, share a limited set of acquired capabilities that define the malignant state [3,4]. Emerging insights into the biology and molecular

signalling pathways of HCC cells have led to the identification of several potential new targets for intervention and the advent of numerous targeted therapies for the treatment of this otherwise lethal tumour. The recent use of Sorafenib, a multikinase inhibitor, is a step forward in the treatment of advanced HCC [5,6], and represents the beginning of a new horizon in the molecular targeted therapy of HCC [7]. Another particularly interesting target is the unlimited replicative potential of cancer cells via the activation of a telomere maintenance mechanism, which is a key step to ensure expansive tumour growth [8].

Telomerase is a ribonucleoprotein complex containing the reverse transcriptase enzyme hTERT that adds repetitive DNA sequences to telomeres, preventing telomere shortening and consequently cell death. Telomerase activity in the adult is present only in a few proliferating cell types, including germ cells, bone marrow stem cells, and epithelial basal cells [9]. In contrast to normal cells, malignant cells from a large variety of human tumours contain active telomerase, which plays a key role in cellular immortalization [10]. In HCC, telomerase reactivation has been detected in over 80% of cases and gene expression analyses have shown that *hTERT* can be included in sets of selected genes that provide molecular signatures, used for the diagnosis and the management of hepatic nodules [11,12]. Moreover, *hTERT* mRNA levels increase during progression of HCCs and correlate with the transition between low- and high-grade dysplastic nodules [13]. Therefore, telomere maintenance and hTERT are considered potential targets for anti-cancer drug development.

The well-known antiviral agent acyclovir (ACV) is a nucleotide analogue acting as a DNA chain terminator [14], mainly used in the treatment of herpes virus infection. The high selectivity of ACV for virus-infected cells is due to the presence of viral thymidine kinases (TK) that specifically and selectively phosphorylate ACV to acyclovir monophosphate (ACV-MP). Human enzymes, guanylate monophosphate (GMP) kinase and nucleoside diphosphate (NDP) kinase, then further phosphorylate ACV-MP to di- (ACV-DP) and triphosphate (ACV-TP), and the latter is incorporated into DNA, arresting its replication [15] (Supplementary Fig. 1). On this basis, adenoviruses carrying the herpesvirus TK gene have been proposed in combination with ACV or its analogues as anti-cancer agents in suicide gene therapy. However, the use of a virus in patients raises ethical concerns, and is undermined by the risk of low and transient expression levels of the transgene [16,17]. We previously have shown in pancreatic cancer the efficacy of acycloguanosyl 5′-thymidyltriphosphate (ACV-TP-T), an ACV-derived pro-drug that is constituted by a thymidine triphosphate attached to the hydroxyl group of ACV [18]. This evidence together with the knowledge of the important role of telomerase in HCC prompted us to test the anti-neoplastic effect of this pro-drug against liver cancer.

In this paper we now provide direct evidence from both *in vitro* analyses and *in vivo* experiments in HCC cells that ACV-TP-T is actually a telomerase substrate, which by incorporating the thymine base in DNA synthesis releases the acyclovir active form. Importantly, we find that ACV-TP-T treatment inhibits tumour growth both in *in vitro* and *in vivo* models of hepatocarcinogenesis.

Materials and methods

Detailed information on the experimental procedures are provided in the Supplementary material and methods.

Results

ACV-TP-T effects on hepatocellular cancer cell lines

Based on the evidence of increased telomerase activity in HCC tissues compared to normal liver [19], human (HepG2, Hep3B, and HuH7) and mouse (Hepa1-6) hepatocellular cancer cell lines were exposed to increasing concentrations of ACV-TP-T (from 0.1 to 1000 μmol/L) for 24 h. ACV-TP-T inhibited DNA synthesis in a dose-dependent manner in all tested cell lines and the calculated half maximal inhibitory concentration (IC_{50}) ranged from 3 to 30 μmol/L (Table 1). Telomerase activity was also tested, and as expected, telomerase activity was present in all liver cancer cell lines analysed and its level was comparable to that of other cancer cell lines from different tissues (data not shown). Interestingly, normal cells derived from healthy human colon, 18CO, showed no presence of telomerase activity and, as expected, ACV-TP-T had no effect on viability or DNA synthesis.

In vitro activation of ACV-TP-T by telomerase

To demonstrate that ACV-TP-T is a telomerase substrate (Supplementary Fig. 1) and that its effect on cancer cell lines depends on its enzymatic activation of the pro-drug, we performed a direct telomerase activity assay using partly purified telomerase from a super-telomerase cell line [20] and decreasing concentrations of ACV-TP-T. Fig. 1A clearly shows that ACV-TP-T is used by telomerase as a substrate in place of dTTP. To investigate, whether telomere synthesis and incorporation of ACV-TP-T by the telomerase releases ACV-DP, the telomere synthesis reaction was analysed by chromatography. The analysis of the telomerase reaction after 6 h of incubation shows that whereas the concentration of ACV-TP-T decreased by about 30%, a new peak representing the formation of about 30% ACV-DP was formed (Fig. 1B). No other products, such as ACV-TP, ACV-MP, or mono-, di- or triphosphorylated dT, were found in appreciable quantities. Taken together, these results confirm that telomerase functions as an activator of the pro-drug, through telomerase-dependent DNA synthesis with a concomitant release of ACV-DP.

Next, we determined the telomerase K_m values for ACV-TP-T and dTTP. We estimated a K_m value for ACV-TP-T of 3.6 mM, which is approximately 50 times higher than the K_m of the reaction with dTTP (Fig. 1C and D). As the physiological concentration of dTTP is in the low micromolar range [21], achieving the

Table 1. Cytotoxicity (based on ATP concentration) and anti-proliferative activity (based on [^{3}H] thymidine incorporation) of ACV-TP-T in four different hepatic cancer cell lines and one normal colon cell line in comparison with telomerase activity.

Cell lines	Viability (IC_{50} μM)	DNA synthesis (IC_{50} μM)	Telomerase activity
Mouse			
Hepa 1-6	>1000	3	260
Human			
HuH7	1000	30	364
Hep-G2	1000	15	410
Hep-3B	1000	8	735
18CO	>1000	>1000	0