

Evaluation of the Potent Anti-Hepatitis B Virus Agent (-)-*cis*-5-Fluoro-1-[2-(Hydroxymethyl)-1,3-Oxathiolan-5-yl]Cytosine in a Novel In Vivo Model

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A murine model was developed to investigate the in vivo activity of anti-hepatitis B virus (HBV) agents. Mice with subcutaneous tumors of HBV-producing 2.2.15 cells showed reductions in levels of HBV in serum and in intracellular levels of HBV when the mice were orally dosed with (-)-*cis*-5-fluoro-1-[2-(hydroxymethyl)-1,3-oxathiolan-5-yl]cytosine (FTC). No effects on tumor size or alpha-fetoprotein levels were observed. FTC can selectively inhibit HBV replication at nontoxic doses.

The majority of in vivo studies of potential anti-hepatitis B virus (HBV) agents have been performed with Pekin ducks infected with duck HBV (7, 12-14, 21, 24), although systems using Beechey ground squirrels and woodchucks infected with ground squirrel and woodchuck hepatitis viruses, respectively, have been developed (16, 23). These systems vary with respect to the involvement of the immune component in the disease. All of these models involve relatively large animals which require substantial quantities of compound for evaluation and are difficult to manage in most laboratories. Also, testing of antiviral agents such as nucleoside analogs could generate aberrant results as a consequence of virus-specific differential susceptibility of the viral polymerase. Nagahata et al. developed a transgenic mouse model expressing HBV which addresses the issues of size and HBV polymerase (15). However, anabolism of nucleoside analogs can differ between human and murine cells. Thus, inhibition of HBV replication could differ between human and murine systems for a given nucleoside analog. We sought to develop a small animal system which provides assessment of anti-HBV selectivity in cells originating from human liver.

The human hepatoma 2.2.15 cell line is often used for in vitro analysis of potential anti-HBV compounds (3, 5, 9, 11, 17, 18). This cell line has integrated HBV genomic sequences, contains all viral DNA replicative intermediates, produces infectious virus (6, 19, 20), and was derived from the well-characterized Hep-G2 cell line (2, 4, 8, 10, 22). To characterize the tumorigenicity of 2.2.15 cells in NIH *bg-nu-xid* mice, 2.2.15 cells (cultured as described by Jansen et al. [9]) were resuspended in RPMI 1640 buffered with 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) at densities of 1.1×10^7 , 3.3×10^7 , or 1.0×10^8 cells per ml. Groups of 4- to 6-week-old female mice (anesthetized by intraperitoneal injections of 100 mg of ketamine and 10 mg of xylazine per kg of body weight) were subcutaneously injected with 100 μ l of cell suspension. Tumor development was monitored for 4 weeks. The mice injected with 10^7 cells developed tumors

greater than 1 mm in diameter within 1 week. However, all animals developed tumors of at least this size after 4 weeks. Ultimate tumor size varied within each group, although average tumor mass was a function of the number of cells injected. No signs of metastasis were detected in any mice with subcutaneous 2.2.15 tumors (data not shown).

To better illustrate the variability of tumor development, tumor progression in five mice injected with 10^7 2.2.15 cells in a separate experiment is shown in Fig. 1. In Fig. 1A, tumor growth was monitored visually. To confirm the presence of 2.2.15 cells within these tumors, blood was collected from the tail veins of the mice at intervals throughout the experiment, and levels of the marker protein, human alpha-fetoprotein (AFP), in sera were determined (Fig. 1B) by using the Tandem E AFP kit (Curtin Matheson Scientific). This protein is secreted specifically by the injected 2.2.15 cells and, therefore, no AFP was detected at the time of injection. Within 1 week, the levels varied from 0.25 to 1.47 μ g/ml, depending on the mouse examined. At final collection, the levels of AFP in serum also varied considerably, with a range of 0.025 to 198 μ g/ml. Overall, AFP levels and ultimate tumor weights correlated well, with correlation coefficients of 0.9 to 1.0. One of the mice injected with 10^7 2.2.15 cells did not have a detectable tumor at 4 weeks postinjection with the hepatoma cells. However, in this mouse, an external tumor was observed at both 1 and 2 weeks postinjection (Fig. 1A), and low levels of circulating AFP (0.11 μ g/ml) were detected at 3 weeks postinjection (Fig. 1B). It is unclear whether this level of AFP was due to a small number of viable 2.2.15 cells or to persistence of AFP in the serum.

We detected HBV in serum samples obtained from mice injected with 2.2.15 cells using an immunoaffinity system linked to quantitative PCR (9). As seen in Fig. 1C, levels of HBV in serum varied extensively for the five tumor-bearing mice and increased dramatically in the late stages of tumor progression. However, correlation between HBV levels and AFP levels or tumor weights is much poorer than that found between AFP levels and tumor weights. For the experiment shown, correlation coefficients of less than 0.75 were found for all but the fourth week, when coefficients were comparable to those found between tumor weights and AFP levels. Similar results have been found in other experiments (data not shown). In contrast

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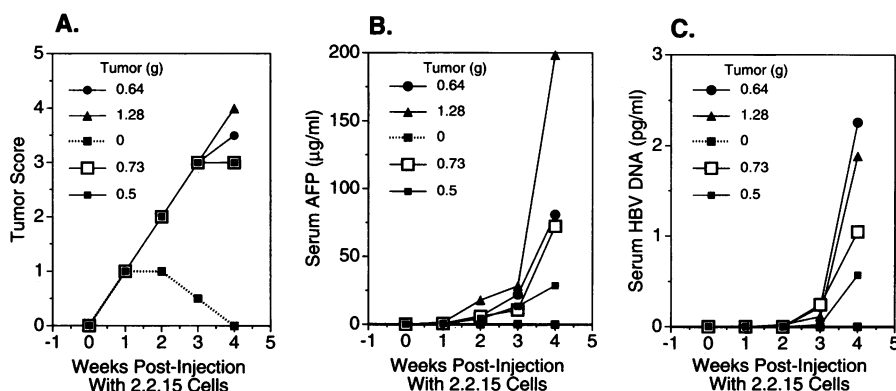


FIG. 1. Time course to establish correlation of levels of AFP and HBV in serum with tumor progression in mice injected with 2.2.15 cells. Tumor progression in five female BNX mice injected subcutaneously with 10^7 2.2.15 cells was monitored by measuring tumor scores visually on the basis of measurements of tumor lengths and widths (1 = 1 to 5 mm; 2 = 6 to 10 mm; 3 = 11 to 15 mm; 4 = 16 to 20 mm; n. 5 = length or width scored in next higher category) (A), levels of circulating AFP (B), and levels of circulating HBV (C).

to human-AFP, HBV was not reliably detected in the serum until 3 weeks postinjection (Fig. 1C). Both delayed detection of circulating virus and decreased correlation between levels of virus in serum and AFP levels or tumor weights (compared with correlation between AFP and tumor weights) might be due to variation in the growth rate of 2.2.15 cells within the tumor. Virus production by the 2.2.15 cells in vitro is quite low in dividing cultures but increases dramatically when the cells are confluent and cell division slows (20). Presumably, as tumor growth rate declines with an increase in size, 2.2.15 cell division also declines and virus production increases.

At the conclusion of the experiment, intracellular viral DNA forms in tumors were examined. Tumor homogenates were incubated for 2 h at 55°C in a lysis buffer consisting of 10 mM Tris-HCl (pH 7.5), 10 mM EDTA, 4 mg of proteinase K per ml, 0.1% sodium dodecyl sulfate, and 300 mM NaCl. Total nucleic acids were extracted twice with 25:24:1 phenol-chloroform-isoamyl alcohol and precipitated with ethanol. Pellets were resuspended in 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, and 18 U of RNA (U.S. Biochemicals, Cleveland, Ohio), the mixtures were incubated for 2 h at 37°C, and the pellets were again extracted with phenol-chloroform-isoamyl alcohol. Thirty micrograms of this DNA was digested with *Nco*I (all restriction enzymes were purchased from New England Biolabs, Beverly, Mass.) and was subjected to Southern blot analyses. *Nco*I cuts once within the HBV (serotype ayw) genome at nucleotide 1374. This region is largely single stranded in the viral relaxed circular molecule but will be cleaved in both integrated and supercoiled forms, generating double-stranded linear DNA. Samples were subsequently electrophoresed and transferred to nitrocellulose for filter blot hybridization (1). Membranes were incubated with a 32 P-labeled RNA complementary to minus-strand HBV DNA representing sequences from the *Eco*RI position at nucleotide 1 to the *Bgl*III position at nucleotide 1986 and were subjected to autoradiography. Signal intensities were quantitated by densitometric scans of X-ray films with a TLC Scanner II (CAMAG Scientific, Inc., Wilmington, N.C.) using CATS3 software. Both integrated and replicative HBV DNA forms were detected (Fig. 2). No significant differences between viral DNA present in tumor extracts and viral DNA extracted from 2.2.15 cells grown in vitro (data not shown) were observed.

To establish that this in vivo system can serve as a model for the evaluation of potential antiviral agents active against HBV, the compound (-) *cis*-5-fluoro-1-[2-(hydroxymethyl)-1,3-oxa-

thiolan-5-yl]cytosine (FTC) (obtained from Dennis Liotta, J. A. Wurster, and Lawrence J. Wilson at Emory University in Atlanta, Ga.) was selected to treat tumor-bearing animals. This antiviral agent shows oral bioavailabilities of >90% in rats and mice dosed with 10 mg/kg (4a) and inhibits HBV replication in the 2.2.15 cell line in vitro at concentrations below 1 μ M (3, 5, 10). In vitro toxicity of this nucleoside analog is minimal (5, 9).

At 1 week postinjection with 10^7 2.2.15 cells, mice were divided into groups of five to begin 3-week dosing with FTC.

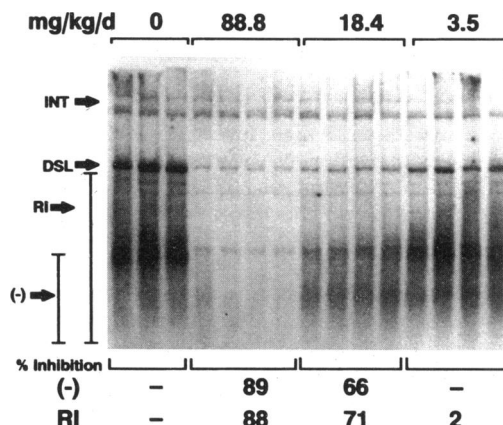


FIG. 2. Effects of FTC on intracellular HBV DNA. Groups of tumor-bearing mice were orally dosed for 21 days with FTC in their drinking water at the indicated concentrations, beginning 1 week postinjection with 2.2.15 cells. Total DNA was extracted from aliquots of harvested tumors, digested with *Nco*I, and analyzed on Southern blots with a probe specific for the 5' region of minus-strand DNA. Each lane contains an equal amount of DNA. Quantitative analysis of the effects on intracellular levels of HBV DNA was performed by densitometric scanning of films. To calculate percent inhibition of single-stranded (ss) HBV DNA of minus polarity in drug-treated mice, the following formula was used: $100\% - \{100 \times [(ss \text{ HBV DNA} / \text{integrated HBV DNA in drug-treated mice}) / (ss \text{ HBV DNA} / \text{integrated HBV DNA in drug-free mice})]\}$. A similar formula was used to calculate the percent inhibition of total replicative-intermediate (RI) HBV DNA in drug-treated mice: $100\% - \{100 \times [(RI \text{ HBV DNA} / \text{integrated HBV DNA in drug-treated mice}) / (RI \text{ HBV DNA} / \text{integrated HBV DNA in drug-free mice})]\}$. INT, integrated HBV DNA; DSL, double-stranded linear HBV DNA. (-), single-stranded HBV DNA of minus polarity.

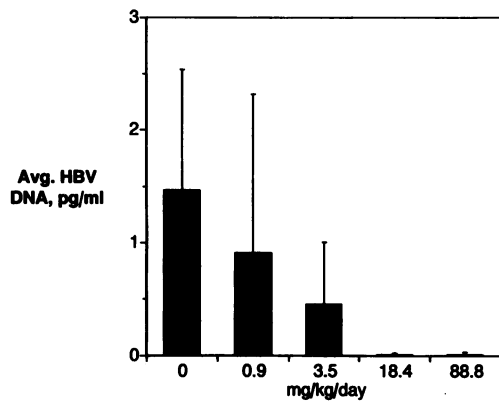


FIG. 3. Effects of FTC on levels of circulating HBV. Groups of tumor-bearing mice were orally dosed for 21 days with FTC in their drinking water at the indicated concentrations, beginning 1 week postinjection with 2.2.15 cells. Levels of HBV DNA in serum were then determined by S antigen capture and PCR analysis (10). Avg., average.

FTC doses (based on group water consumption) included 0.9, 3.5, 18.4, and 88.8 mg/kg/day. Nine mice served as controls and received no drug. As described above, tumor progression was monitored visually and by determination of the amount of AFP in serum. Levels of circulating HBV were determined throughout the experiment. Tumor progression and AFP levels varied in the characteristic pattern shown in Fig. 1, indicating that the selected doses of FTC did not affect tumor development (data not shown). When tumor extracts were examined on Southern blots, replicative viral DNA forms were detected for all groups of mice; however, there was a marked reduction in intracellular levels of replicative HBV DNA in the animals treated with 18.4 and 88.8 mg/kg/day of FTC (Fig. 2). Interestingly, the double-stranded linear DNA form was reduced in tumors extracted from mice treated with high levels of FTC. In addition, circulating levels of HBV DNA were significantly reduced in mice given 18.4 and 88.8 mg of FTC per kg per day (Fig. 3). Although some effect on the levels of circulating virus may have occurred at lower doses of FTC, variation among individuals makes this unclear.

The ability to detect HBV replication and human AFP (Fig. 1) in mice with subcutaneous tumors of 2.2.15 cells provides an opportunity to evaluate the efficacy of anti-HBV agents in a novel small-animal model. Both FTC and carbocyclic 2'-deoxyguanosine (CDG) are potent and highly selective in cultured 2.2.15 cells (3, 5, 9, 11, 17, 18). Although CDG has been shown to suppress duck HBV replication in chronically infected ducks (14), we were unable to identify an efficacious, nontoxic dose of CDG in this model (data not shown). In contrast, FTC strongly inhibited replication of HBV within the tumors (Fig. 2), and circulating virus titers were much less than those of control, drug-free mice (Fig. 3). The doses used did not produce any signs of toxicity to the mice or effects on tumor size and AFP levels (data not shown), indicating that FTC selectively inhibited HBV production by the 2.2.15 tumors in these mice. Recently, this drug has been used in an additional animal model; FTC suppressed woodchuck hepatitis virus replication in naturally infected woodchucks (23a).

At higher doses of FTC, levels of double-stranded linear viral DNA generated by *Nco*I digestion of integrated and protein-free DNA extracted from tumors (Fig. 2) were much lower than those observed in extracts from drug-free mice. This implies that FTC is affecting levels of supercoiled viral

DNA within the tumors in a relatively short time period (21 days). One mechanism by which this could occur is inhibition of production of relaxed circular genomic viral DNA, which is thought to cycle back to the nucleus to generate supercoiled viral DNA. Regardless of the mechanism, these data suggest that if supercoiled viral DNA has a finite half-life, then long-term treatment with FTC has the potential to cure cells of the nuclear episomal DNA template.

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