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Inhibition of Replication of Hepatitis B Virus by Cytallene In Vitro

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The acyclic cytosine nucleoside analog cytallene [1-(4′-hydroxy-1′,2′-butadienyl)cytosine], which has both (+)- and (−)-enantiomers, was evaluated for its anti-hepatitis B virus (HBV) activity in 2.2.15 cells and was found to have potent activity against HBV DNA synthesis. The *R*-(−)-enantiomer was found to be the more active of the cytallene enantiomers, with a 50% inhibition concentration against HBV synthesis ($HBIC_{50}$) of 0.08 μM. Its antiviral activity could be reversed by deoxycytidine (dC) and less efficiently by cytidine. Upon removal of the *R*-(−)-enantiomer from culture medium, the synthesis of HBV DNA could reinitiate, which suggested that the antiviral action is reversible. The *R*-(−)-enantiomer was also found to be more cytotoxic than the *S*-(+)-enantiomer. The degree of cytotoxicity varied among the cell lines, with a 50% inhibition of cell growth at greater than 10 μM. The *R*-(−)-enantiomer had no effect on HBV RNA synthesis and mitochondrial DNA synthesis at a concentration of 10 times or more than the $HBIC_{50}$. The two enantiomers cannot be deaminated by dC deaminase, and they can be phosphorylated by cytoplasmic dC kinase. The *R*-(−)-enantiomer of cytallene is the first acyclic cytosine analog with potent inhibitory activity against HBV similar to those of other L-(−)-ddC analogs.

Hepatitis B virus (HBV) infection is a major health problem around the world. The infection not only causes acute and chronic hepatitis, which could lead to liver cirrhosis, but it also has a strong association with hepatocellular carcinoma (1, 12). An effective anti-HBV drug would be useful for the treatment of HBV-associated hepatitis and might be useful for preventing or delaying the onset of hepatocellular carcinoma.

Recently, several L-nucleoside analogs have been discovered to have potent anti-HBV activity in cell culture and in animal models (2, 6, 8, 13, 17, 19–22, 26). A majority of these L-nucleosides are L-dideoxycytidine (L-ddC) analogs and are also potent inhibitors of human immunodeficiency virus (HIV). The only exception to this is the thymidine analog 2′-fluoro-5-methyl-β-L-arabinofuranosyluracil (L-FMAU), which has anti-HBV and anti-Epstein-Barr virus activity (6, 22). All of those potent anti-HBV L-nucleoside analogs have low levels of activity against cell growth in culture, which suggests the possible lack of acute toxicity in patients at antiviral dosages, and also have low levels of activity against cellular mitochondrial DNA (mtDNA) synthesis in culture, which suggests the possible lack of chronic toxicity in patients at anti-HBV dosages upon long-term treatment (8). One of those L-nucleosides, 2′,3′-dideoxy-3′-thiacytidine [L(−)SddC, or 3TC], has been approved for clinical usage in combination with zidovudine for the treatment of patients with AIDS (10). L(−)SddC is also undergoing a phase III clinical trial for the treatment of HBV infection, and the preliminary clinical results look very promising (7).

Cytallene, a unique cytosine analog, was synthesized and found to have potent activity against HIV in culture (14), with the *R*-(−)-enantiomer more potent than the *S*-(+)-enantiomer (16). In this study, we report the potent activity of the *R*-(−)-enantiomer of cytallene against HBV as well as its lack of activity against mtDNA synthesis in cells. This is the first cytosine analog with allene substituting for ribose or deoxyribose to have both anti-HBV activity and anti-HIV activity at concentrations which have no effect on either nuclear DNA or mtDNA synthesis of cells.

MATERIALS AND METHODS

Compounds and chemicals. Compounds 1-(4-hydroxy-2-butynyl)cytosine (compound 1b), 5-fluoro-1-(4′-hydroxy-2′-butynyl) cytosine (1c), (±)-5-fluorocytallene (1e), and (±)-cytallene (1d) and its *S*-(+)- and *R*-(−)-enantiomers (3 and 2) were synthesized by B. C. N. M. Jones, C. Simons, and J. Zemlicka (Wayne State University, Karmanos Cancer Institute, Detroit, Mich.) and were at least 95% pure, as shown by nuclear magnetic resonance spectra and chiral high-performance liquid chromatography (HPLC) (16, 23, 24). 2′,3′-ddC (compound 1a) was purchased from Pharmacia. Compounds 1b and 1a were dissolved in phosphate-buffered saline (0.8% NaCl, 0.02% KCl, 10 mM Na_2HPO_4, 1.76 mM KH_2PO_4 [pH 7.4]), and the other compounds were dissolved in dimethyl sulfoxide.

Assay for antiviral activity in vitro. The 2.2.15 cells were kindly provided by G. Acs (Mount Sinai Medical Center, New York, N.Y.). Cells were maintained in minimal essential medium (MEM) supplemented with 10% fetal bovine serum and incubated at 37°C in a moist atmosphere containing 5% CO_2–95% air. The cells were inoculated in tissue culture plates at a density of 5×10^4 cells per ml of MEM in 3.83-cm^2 wells. The medium was changed every 3 days after inoculation. On days 6 and 9, the compounds studied were added to the medium. Cells were incubated with various concentrations of compounds for 6 days. The extracellular DNA from the culture medium was isolated and processed (8). HBV-specific hybridization was done as described earlier (8, 22). The intensity of the autoradiographic bands was quantitated with a scanning densitometer (Molecular Dynamics). The values were obtained by plotting the percentage of inhibition compared with that of the control versus the drug concentration.

Isolation and characterization of intracellular DNA. Cells harvested from cultures treated with the compounds as well as untreated controls were lysed in 0.5 ml of lysis buffer with proteinase K at 0.1 mg/ml. Intracellular DNA was isolated and processed for Southern analysis as described earlier (8, 22). After hybridization to an HBV-specific probe, the inhibitory effect on HBV DNA synthesis was ascertained by normalization to the signal of integrated HBV DNA (8, 22).

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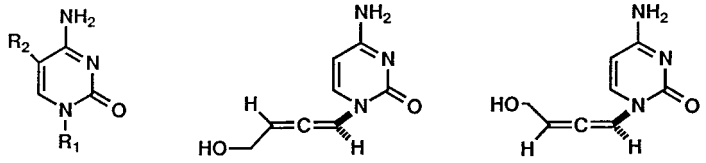
TABLE 1. Comparative potencies of ddC and cytallene analogs as monitored by anti-HBV, cytotoxicity, and mtDNA effects



1 2 3

Compound[a]	$HBIC_{50}$ (μM)	$mtIC_{50}$ (μM)	EC_{50} (μM)	SI[b]
ddC (1a)	1.4	0.07	10	7
Butynol 1b	>100	>100	>100	NA
(±)-Cytallene (1d)	0.12	>50	>30	NA
R-(−)-Cytallene (2)	0.08	>10	12	150
S-(+)-Cytallene (3)	>10	>50	>30	NA
(±)-5-Fluorocytallene (1e)	62	>100	>100	NA
Butynol 1c	100	>100	>100	NA

[a] The compound numbers are shown in parentheses. Compounds: 1a, R_1 = 1-β-D-2′,3′-dideoxyribofuranosyl, R_2 = H; 1b, R_1 = CH_2-C≡C-CH_2OH, R_2 = H; 1c, R_1 = CH_2-C≡C-CH_2OH, R_2 = F; 1d, R_1 = CH=C=CH-CH_2OH, R_2 = H; 1e, R_1 = CH=C=CH-CH_2OH, R_2 = F.

[b] SI, selective index ($EC_{50}/HBIC_{50}$); NA, not applicable.

Analysis of the effects of cytallene on HBV-specific RNA. The 2.2.15 cells were inoculated at a density of 3×10^5 cells per 5 ml of MEM in 25-cm^2 flasks. The medium was changed every 3 days after inoculation, and from day 6 to day 12, the cells were treated with 4 μM (±)- or *R*-(−)-cytallene. On day 12, total cellular RNA was isolated (5). Northern analysis of the RNA was done, and HBV-specific transcripts were identified (22). Normalization was done by hybridization to the transcript of a human glyceraldehyde-3-phosphate-dehydrogenase probe.

Detection of HBsAg. For detection of hepatitis B surface antigen (HBsAg), culture medium from drug-treated cultures was assayed for the presence of HBsAg before the cells were harvested. An aliquot of the culture medium was appropriately diluted and assayed for HBsAg (22). Comparisons of HBsAg levels were made with respect to drug-treated versus untreated cultures.

Determination of mtDNA content. CEM (human T lymphoblastoid) cells were seeded in 2 ml of RPMI 1640 medium containing 10% dialyzed fetal bovine serum at a concentration of 2×10^5 cells per ml. The cells were incubated with various concentrations of the compounds for 4 days, and then the cells were counted, centrifuged (3 min, 1,000 × *g*), and resuspended to a density of 2×10^5 cells per ml with fresh medium plus drugs. On day 6, the cells were counted, centrifuged, and resuspended again as described above. On day 8, 10^5 cells were collected by centrifugation and processed for isolation of DNA and for slot blot analysis (8). The mtDNA was detected with an mtDNA-specific probe (3). $mtIC_{50}$ was defined as the drug concentration causing a 50% reduction in mtDNA content. The impact of the various compounds on the mtDNA was also ascertained by hybridization of the Southern blots of DNA from 2.2.15 cultures treated with the drug. This was done after removal of the HBV-specific probe and rehybridization to the mtDNA-specific probe.

Determination of lactic acid production in cultures treated with *R*-(−)-cytallene. Since a number of antiviral nucleosides such as ddC and 1-(2′-deoxy-2′-fluoro-β-D-arabinofuranosyl)-5-iodouracil (FIAU) interfere with mitochondrial function, we wanted to test the effect of *R*-(−)-cytallene on mitochondrial function. Lactic acid production, which is a reflection of aberrant mitochondrial function, was used as the end point. HepG2 cultures were treated with *R*-(−)-cytallene at 24 μM on days 1, 3, and 6 as described earlier (22). On day 8, an aliquot of the culture medium was processed for determination of lactic acid (22). ddC at 20 μM and untreated controls were maintained along with the cytallene treatments.

Cytotoxicity. CEM cells or dC kinase (dC K)-deficient CEM cells were seeded in RPMI 1640 medium containing 10% dialyzed fetal bovine serum at a density of 10^4 cells per ml of medium. The generation time of CEM cells is approximately 20 h. After a 24-h incubation, compounds were added in various concentrations. The cells were incubated with the compounds for 60 h, after which time cell number was determined with a Coulter Counter and indirectly by an MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) dye reduction method (15). EC_{50} was defined as the drug concentration that caused a 50% reduction in cell number.

Susceptibility to dC deaminase. The reaction mixture containing 25 mM Tris-HCl (pH 7.5), 0.4 mM cytallene, and dC deaminase in a final volume of 50 μl was incubated at 37°C for 16 h. dC deaminase was purified from human liver through DEAE-cellulose fast-performance liquid chromatography with Mono Q and Mono S columns by a previously published procedure (19). The reaction was terminated by addition of 100 μl of acetonitrile. The precipitated protein was removed by centrifugation, and the supernatant was lyophilized to dryness. The sample was reconstituted with the eluting buffer (5% methanol) and analyzed by HPLC with an Alltech RP-C_{18} column.

Phosphorylation of cytallene by dC kinase. The reaction mixture containing 60 mM Tris-HCl (pH 7.5), 25 mM NaF, 0.16 mM tetrahydrouridine (obtained from the National Institutes of Health, Bethesda, Md.), 1.8 mM phosphocreatine, creatine phosphokinase, 5.6 mM ATP-$MgCl_2$, 0.4 mM cytallene, and 0.01 U of cytoplasmic dC K or 0.0006 U of mitochondrial deoxypyrimidine nucleoside kinase (dpyrd K) in a final volume of 50 μl was incubated at 37°C for 2 h (20 h for mitochondrial dpyrd K). Cytoplasmic dC K was purified from BL21(de3) bacteria carrying the PET-3d expression vector, into which the cDNA of the human cytoplasmic dC gene was cloned (4). The purified enzyme had a specific activity of 13 U/mg. Mitochondrial dpyrd K was purified from human chronic lymphocytic leukemia cells by procedures described previously (18) and had specific activity greater than 6 U/mg. The reaction was terminated by addition of 200 μl of methanol. The precipitated protein was removed by centrifugation, and the supernatant was lyophilized to dryness. The sample was reconstituted with water and analyzed by HPLC with a Whatman Partisil 10 SAX column.

dC K and mitochondrial dpyrd K inhibition by cytallene. The assay procedures were the same as those described previously (4, 18), with the exception that 25 μM [^{14}C]dC was used in the presence of 588 μM enantiomers of cytallene. The reaction mixture was incubated at 37°C for 2 h.

RESULTS

Anti-HBV activity of cytallene and its analogs in vitro. The structures of ddC (compound 1a), butynols 1b and 1c, (±)-5-fluorocytallene(1e), and (±)-cytallene (1d) and its S-(+)- and R-(−)-enantiomers (3 and 2) used in this study are shown in Table 1. The 2.2.15 cell line was used to evaluate the anti-HBV activity of cytallene and its analogs. Our studies showed that (±)-cytallene and *R*-(−)-cytallene had potent anti-HBV activity, but the (+)-enantiomer did not show anti-HBV activity up to 10 μM. The antiviral effects were measured by analysis of extracellular and intracellular HBV DNA (Fig. 1). The results indicated that the amount of extracellular HBV DNA decreased in a dose-dependent manner. The 50% inhibition concentration against HBV synthesis ($HBIC_{50}$) values of (±)-cytallene and *R*-(−)-cytallene are presented in Table 1. Replication of HBV DNAs in 2.2.15 cells treated with various concentrations of (±)-cytallene and *R*-(−)-cytallene were also examined. Intracellular DNAs were digested with the restriction enzyme *Hin*dIII, which does not cleave the HBV genome, and subjected to Southern blot analysis with a ^{32}P-labeled HBV DNA as a probe. The episomal DNA and the chromosomally integrated HBV DNA genomes were separated in the gel and can be differentially quantified. The amount of episo-

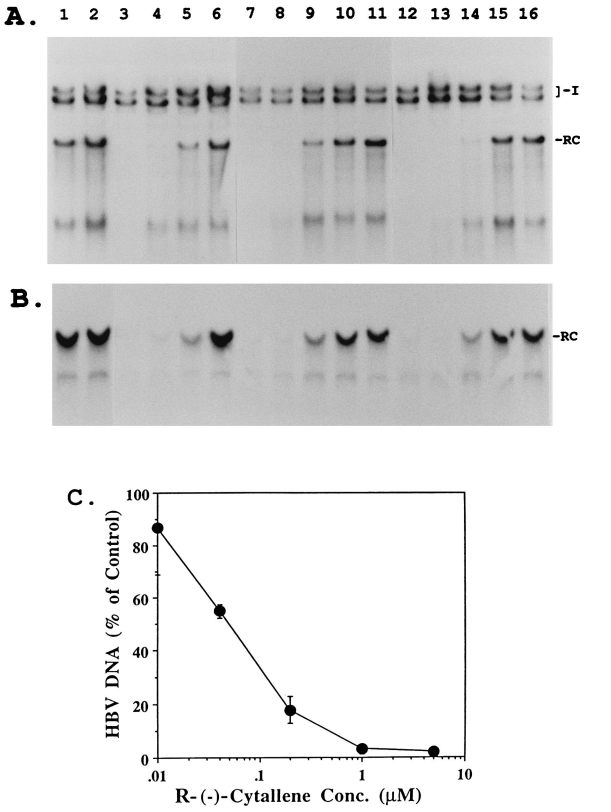


FIG. 1. HBV inhibition by ddC, (±)-cytallene and *R*-(−)-cytallene. Hybridization to a HBV probe was done for samples isolated from intracellular DNA (A) and culture medium (B). The 2.2.15 cells were untreated (lanes 1 and 2) or treated with ddC at 50, 10, 2, and 0.4 μM (lanes 3, 4, 5, and 6, respectively); (±)-cytallene at 5, 1, 0.2, 0.04, and 0.01 μM (lanes 7, 8, 9, 10, and 11, respectively); and *R*-(−)-cytallene at 5, 1, 0.2, 0.04, and 0.01 μM (lanes 12, 13, 14, 15, and 16, respectively). The positions of integrated (I) and relaxed circular (RC) HBV DNA are shown on the right. The DNA was processed and analyzed as described in Materials and Methods. Dose-dependent inhibition of HBV DNA from cultures treated with various concentrations of *R*-(−)-cytallene is also shown (C).

mal HBV DNA decreased in a dose-dependent manner, similar to the inhibition seen on extracellular HBV DNA, and both the amount and the restriction enzyme pattern of the chromosomally integrated HBV genome were unaltered (Fig. 1A). The results clearly indicated that the cytallene can effectively lower the HBV DNA levels by inhibiting DNA replication. The (−)-enantiomer is more potent than the (+)-enantiomer–(−)-enantiomer mixture.

Reversibility of anti-HBV action by (±)-cytallene and *R*-(−)-cytallene. The 2.2.15 cells, which were treated with 0.5 μM cytallene or *R*-(−)-cytallene for 21 days, were incubated for an additional 12 days in the absence of the compounds, and the medium was changed every 3 days. The results indicated that HBV DNA could be identified extracellularly after 6 days of drug-free incubation, although not to the same extent as those in untreated control cells, and the amount of extracellular HBV DNA increased in a time-dependent manner for both (±)-cytallene and *R*-(−)-cytallene. After 12 days of drug-free incubation, the extracellular HBV DNA levels of drug-treated cultures were at a level equal to that of the untreated control (Fig. 2). This suggests that the continued presence of the nucleoside is essential for the maintenance of inhibitory activity.

Reversal of *R*-(−)-cytallene anti-HBV action by dC and less efficiently by cytidine. The 2.2.15 cells were treated with 1 μM *R*-(−)-cytallene alone or in combination with various concentrations of cytidine or dC, respectively, for 6 days. As shown in Fig. 3, the amount of extracellular HBV DNA increased in a dose-dependent manner for dC-added, *R*-(−)-cytallene-treated cells. At 100 μM, dC could completely reverse *R*-(−)-cytallene anti-HBV action. In contrast, combination treatment of *R*-(−)-cytallene with cytidine did not affect the anti-HBV action of *R*-(−)-cytallene.

Comparative potency of cytallene analogs as inhibitors of HBV in vitro. Various analogs of cytallene (compound 1d), compounds 2 and 3, butynols 1b and 1c, and (±)-5-fluorocytallene (1e) were tested in 2.2.15 cells to evaluate their anti-HBV activity. The $HBIC_{50}$ values of these compounds are presented in Table 1. Analog 1e, with an F substitution at the 5 position, had much weaker anti-HBV activity than that of cytallene (1d), with an EC_{50} of 62 μM. However, the compounds 1b and 1c, with a triple bond in the side chain, did not show any anti-HBV activity at 100 μM. A similar pattern was observed in the anti-HIV activity (14, 24).

Effect on HBV-specific RNA and HBsAg. Even though *R*-(−)-cytallene produced pronounced inhibition of HBV DNA synthesis, no effects on HBV-specific transcription were detected. Three major HBV transcripts of approximately 3.5, 2.5, and 2.1 kilobases were detected in the total cellular RNA. When the transcript levels were normalized with the amount of glyceraldehyde-3-phosphate dehydrogenase mRNA, no significant differences could be observed in the levels of HBV-specific transcripts by 4.0 μM *R*-(−)-cytallene (data not shown). Lack of inhibition of virus-specific transcripts is due to

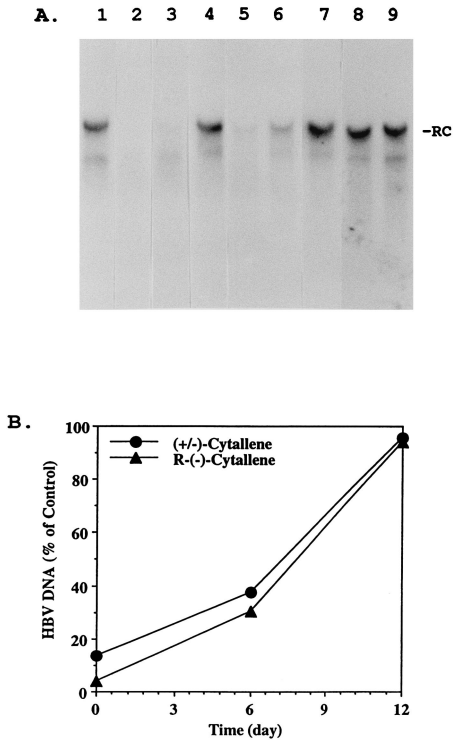


FIG. 2. Reversibility of (±)-cytallene and *R*-(−)-cytallene action on HBV replication. The 2.2.15 cells either untreated (lanes 1, 4, and 7) or treated with 0.5 μM (±)-cytallene (lanes 2, 5, and 8) or *R*-(−)-cytallene (lanes 3, 6, and 9) for 21 days were incubated with drug-free medium for 0 (lanes 1, 2, and 3), 6 (lanes 4, 5, and 6) or 12 (lanes 7, 8, and 9) days (A). HBV-specific DNA in the medium was analyzed as described in Materials and Methods. RC, relaxed circular HBV DNA. The time-dependent curve is also shown (B).

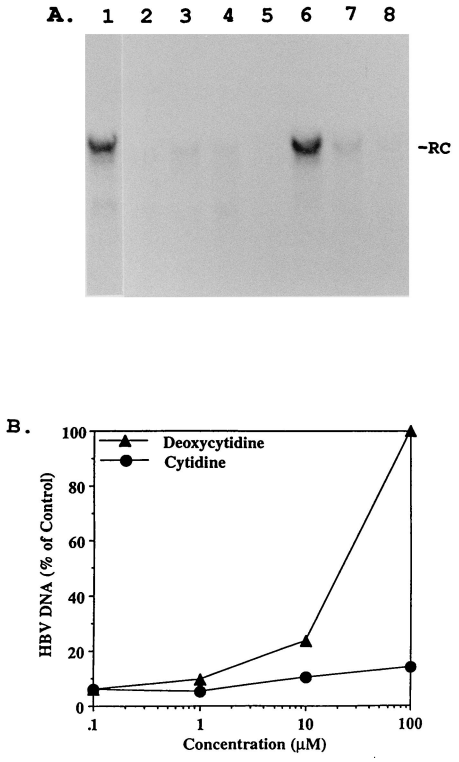


FIG. 3. Reversal of *R*-(−)-cytallene anti-HBV action by dC. The 2.2.15 cells either were untreated (lane 1) or were treated with 1 μM *R*-(−)-cytallene alone (lane 2); 1 μM *R*-(−)-cytallene combined with 100, 10, and 1 μM cytidine (lanes 3, 4, and 5); or 100, 10, and 1 μM dC (lanes 6, 7, and 8) (A). HBV-specific DNA in the medium was analyzed as described in Materials and Methods. The dose-dependent curve is also shown (B).

the inability of the cytallene triphosphate to inhibit the host RNA polymerase II, which transcribes the HBV DNA. The *R*-(−)-cytallene also had no effect on HBsAg in culture medium of 2.2.15 cells even at 10 μM (data not shown). This is consistent with the observation of a lack of effect on HBV RNA transcription.

Effect on mtDNA content and cytotoxicity. The $mtIC_{50}$s of the compounds are shown in Table 1. There was no adverse effect on mtDNA synthesis at concentrations of *R*-(−)-cytallene that caused cessation of HBV replication. In contrast, ddC had a low $mtIC_{50}$ of 0.07 μM, which was even lower than its $HBIC_{50}$ of 1.4 μM. The EC_{50}s of the compounds are shown in Table 1. The *R*-(−)-cytallene is more toxic than *S*-(+)-cytallene against CEM cell growth, with an EC_{50} of 12 μM, which is much higher than its $HBIC_{50}$. Analysis of the mtDNA in 2.2.15 cells showed that there was no appreciable reduction in the mtDNA in cultures treated with *R*-(−)-cytallene up to 10 μM, whereas ddC was a potent inhibitor of mtDNA synthesis (Fig. 4A). Also HepG2 cultures treated with ddC at 20 μM showed significant amounts of lactic acid production, whereas cells treated for the same period of time with 24 μM *R*-(−)-cytallene showed low levels comparable to those in untreated controls (Fig. 4B). The levels of lactic acid in the control as well as *R*-(−)-cytallene cultures were not significant, because they were below the levels of accurate determination by the methodology used. This suggests that *R*-(−)-cytallene has a selective advantage over ddC when being considered for development as an anti-HBV drug. When the cytotoxicity of *R*-(−)-cytallene was evaluated against dC K-deficient CEM cells, the EC_{50} was about five times higher. In contrast, dC K-deficient CEM cells were at least 100 times less sensitive than CEM cells to cytosine arabinoside (AraC) (Fig. 5).

Phosphorylation by dC K and susceptibility to dC deaminase. Both *S*-(+)-cytallene and *R*-(−)-cytallene were phosphorylated by cytoplasmic dC K with relative V_{max}/K_m values of 0.12 and 0.15, respectively. The *R*-(−)-cytallene had a lower K_m value and V_{max} value than *S*-(+)-cytallene (Table 2). Similar V_{max}/K_m values were observed for 5-fluoro derivatives of D- and L-ddC (11). On the other hand, neither *S*-(+)- nor *R*-(−)-cytallene was phosphorylated by mitochondrial dpyrd K under the conditions we studied. The poor substrate behavior of *R*-(−)-cytallene toward dpyrd K is further supported by the observation that *R*-(−)-cytallene at a 20-fold concentration of dC in the reaction mixture did not inhibit mitochondrial dpyrd K.

With dC used as a control, 100% of dC was converted to deoxyuridine in 3 h by human dC deaminase. However, no deamination was observed for either (±)-, *S*-(+)-, or *R*-(−)-cytallene after a 24-h reaction, which indicated that cytallene was resistant to the deamination by human dC deaminase. Previously, it was found that (±)-cytallene is not a substrate for bovine cytidine deaminase (23), but it was phosphorylated in a reaction catalyzed by dC kinase from human leukemic spleen cells (9).

DISCUSSION

Cytallene is a cytosine analog with ribose substituted by the 4′-hydroxy-1′,2′-butadienyl group, which has a potent activity against HIV in culture (14). It is composed of two enantiomers. In this report, cytallene is also shown to be active against HBV in culture, with the *R*-(−)-enantiomer being the active form. The concentration of *R*-(−)-cytallene required for anti-HBV activity is much lower than that required to inhibit cell growth or mtDNA synthesis. This feature and its spectrum of activity are similar to those of other L-(−)-ddC analogs which were recently shown to have potent anti-HBV and anti-HIV activity in cell culture (20, 21). The selective index of the *R*-(−)-enantiomer is lower than that of 3TC. However, there is

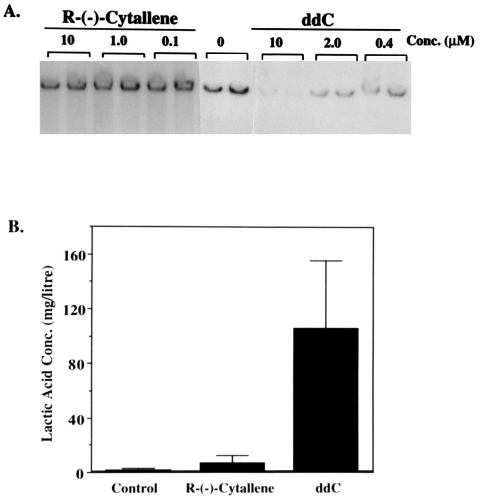


FIG. 4. Effects of *R*-(−)-cytallene on mtDNA content and lactic acid production. (A) Southern blots that were hybridized to the HBV-specific probe were rehybridized to the mtDNA-specific probe cytochrome *c* oxidase III. (B) Lactic acid production of cultures treated with *R*-(−)-cytallene (24 μM), ddC (20 μM), or untreated controls were determined with the lactic acid estimation kit as per the manufacturer's instructions. The means ± standard deviations of three independent cultures are represented.

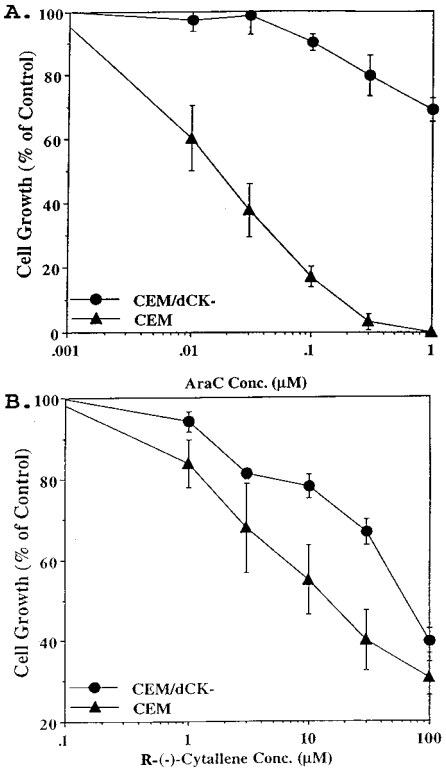


FIG. 5. Growth inhibition of cytosine arabinoside (AraC) (A) and *R*-(−)-cytallene (B) on CEM cells and dC K-deficient CEM cells.

a major difference in structure-activity relationship of cytallene versus that of the D- and L-enantiomers of 2′,3′-ddC or SddC (3TC). For L-ddC and L-SddC, the substitution of the 5-hydrogen of cytosine by a fluoro atom could either increase or cause no alteration of their anti-HBV activity (8, 20), whereas cytallene is more active than 5-fluorocytallene. The reason for such a difference in structural requirements in the group of active anti-HBV cytosine analogs is not clear. Nevertheless, a similar detrimental effect of the fluorine atom in 5-fluorocytallene on anti-HIV efficacy was also observed (24). When the anti-HBV activities of different cytallene analogs were examined, the results clearly indicated the importance of axial chirality of the allene component for cytallene antiviral activity. The emergence of HBV resistant to L(−)SddC in HBV-infected patients treated with L(−)SddC was recently reported (25). Given the difference between the structure requirements of anti-HBV activity of L(−)SddC and *R*-(−)-cytallene, it is conceivable that *R*-(−)-cytallene may be active against L(−)SddC-resistant HBV. This will be further investigated.

The anti-HBV action of *R*-(−)-cytallene is reversible. This is similar to what has been found for other anti-HBV L-ddC analogs (8), as is the reversibility of the anti-HIV effect (15). Long-term usage of this compound in the treatment of HBV infection will be needed if this compound is ever developed as an anti-HBV drug in clinics. However, the results from the in vitro studies cannot be directly extrapolated to humans, because the 2.2.15 cells have the HBV DNA integrated into the cells.

Our investigations of the ability of dC and cytidine to relieve the anti-HBV effects of cytallene showed that dC could decrease *R*-(−)-cytallene anti-HBV activity much more efficiently than cytidine. The mechanism of reversal by dC of the anti-HBV effect when used in combination with cytallene is likely due to competition of phosphorylation of *R*-(−)-cytallene in cells and/or reversal of the action of *R*-(−)-cytallene metabolites at target sites, e.g., HBV DNA polymerase. The *R*-(−)-cytallene could be phosphorylated by cytoplasmic dC K but not by mitochondrial dpyrd K. The important role of cytoplasmic dC K in phosphorylation of *R*-(−)-cytallene is supported by the observation that dC K-deficient CEM cells are less sensitive to the compound than CEM cells. However, *R*-(−)-cytallene may still be phosphorylated by other nucleoside kinases. This will require further investigation, since dC K-deficient CEM cells are only about five times less sensitive than CEM cells to this compound. *R*-(−)-Cytallene is resistant to dC deaminase action. It is a substrate of cytoplasmic dC K. The K_m value of *R*-(−)-cytallene is about the same as that of dC for cytoplasmic dC K, whereas the V_{max} is about 15% compared with that for dC. Thus, the decrease in anti-HBV activity of *R*-(−)-cytallene by dC could not be solely due to the competition of *R*-(−)-cytallene phosphorylation by dC alone, since a high concentration of dC is required to reverse the anti-HBV activity of *R*-(−)-cytallene. The mechanism of anti-HBV action of *R*-(−)-cytallene is due to its ability to inhibit HBV DNA synthesis but not HBV RNA or protein synthesis. The target is likely to be HBV DNA polymerase. The mechanism of decreasing anti-HBV activity of *R*-(−)-cytallene by dC could be primarily due to the reversal of *R*-(−)-cytallene triphosphate action on HBV DNA polymerase by dCTP. This will require further investigation. It was also noted that *R*-(−)-cytallene can act as a substrate with the same efficiency as *S*-(+)-cytallene for cytoplasmic dC K, whereas *R*-(−)-cytallene is much more potent than *S*-(+)-cytallene in inhibiting HBV. Thus, the differences between the anti-HBV activities of *R*-(−)- and *S*-(+)-cytallene are due to differential behavior of these compounds or their metabolites toward other biochemical determinants. It will be of interest to compare their metabolisms and the actions of their triphosphate metabolites on HBV DNA polymerase. The metabolism studies would require the radioactive *R*-(−)- and *S*-(+)-cytallene, which are not currently available.

TABLE 2. Phosphorylation of *S*-(+)- and *R*-(−)-cytallene by cytoplasmic dC K or mitochondrial dpyrd K

Compound	Cytoplasmic dC K		Mitochondrial dpyrd K	
	V_{max}[a]	K_m (μM)	V_{max}[a]/K_m	V_{max}[a]
dC	1	7	1	1
R-(−)-Cytallene	0.16	9	0.12	>0.1
S-(+)-Cytallene	1.07	50	0.15	>0.1

[a] Relative V_{max}.

In summary, *R*-(−)-cytallene is a potent anti-HBV cytosine analog with a unique structure, which is not shared with other anti-HBV L-ddC compounds, whereas the spectrum of its antiviral activity and the mechanism of action are similar to those of other L-ddC analogs. The future of this compound as a potential clinical candidate for the treatment of HBV infection will depend on whether this unique structure of *R*-(−)-cytallene could translate into unique activity which is not shared with other anti-HBV L-ddC compounds.

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