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Inhibitory Activity of New Nucleoside Analogs on Wild Type and YMDD Mutants of The Hepadnavirus Reverse Transcriptase

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The major problem of lamivudine therapy of chronic hepatitis B is the selection of resistant mutants of the HBV polymerase. In an attempt to design combination strategies for the treatment of chronic hepatitis B, we have evaluated the inhibitory activity of novel compounds : (-) FTC, L-FMAU, DXG and DAPD on the hepadnavirus reverse transcriptase, *in vitro*. The activity of these nucleoside analog triphosphates (-TP) was investigated in an *in vitro* assay for the expression of an enzymatically active wild type DHBV polymerase. DAPD-TP was found to be the more active compound on the incorporation of nucleotides in viral minus strand DNA with an IC50 of 0.7 ± 0.2 μM, followed by 3TC-TP (6.1 ± 3.5 μM), FTC-TP (8.5 ± 4.1 μM) and DXG-TP (16.9 ± 11.1 μM), while L-FMAU-TP was a weak inhibitor of the RT activity. Moreover, DAPD-TP was found to inhibit the priming of RT, while DXG-TP was a weak inhibitor of priming. Using mutants in the bulge of epsilon, we could demonstrate that DAPD-TP, FTC-TP and 3TC-TP are likely to be DNA chain terminator. Cell culture experiments also showed that L-FMAU is a weak RT inhibitor but may mainly inhibit the DNA dependent DNA polymerase activity of the HBV polymerase. Then, the activity of these compounds on lamivudine resistant mutants was assessed. Mutants of the B and C (YMDD motif) domains of the viral RT were expressed *in vitro* (M512I, M512V, and L489M+M512V). The IC50 of 3TC-TP and FTC-TP increased dramatically (> 100 μM) indicating that these mutants are cross-resistant to these cytidine analog triphosphates. Interestingly, the double polymerase mutant remained sensitive to DXG-TP and DAPD-TP with an IC50 of 30.9 ± 20.6 μM and 13.1 ± 8.4 μM respectively.

Altogether these promising results indicate that DXG and DAPD should be further evaluated in experimental models to assess the best combinations for the treatment of chronic hepatitis B and the prevention of selection of resistant mutants.

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Comparison of the Anti-HBV Activities of Lamivudine, Adefovir, Famciclovir, Lobucavir, and Emtricitabine Against Wild-type and Lamivudine-Resistant HBV Polymerases *in Vitro*.

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Lamivudine, adefovir, famciclovir, lobucavir, and emtricitabine are nucleoside or nucleotide analogs with antiviral activity against hepadnaviruses *in vivo* and *in vitro*. Previously published IC_{50} values for these 5 compounds in HBV cell culture assays did not correlate exactly with their anti-HBV efficacy *in vivo* comparing between different clinical trials. To directly compare the activities of these compounds *in vitro*, we determined the inhibition constants for the triphosphates of these compounds in enzymatic assays using recombinant wild-type HBV polymerase. Our results indicated that the potency of these compounds was in the order of adefovir = lobucavir > lamivudine = emtricitabine >> famciclovir.

Lamvidine demonstrated good anti-HBV activity *in vitro* and has been used widely for treating HBV infections *in vivo*. However, long-term treatment with lamivudine resulted in the emergence of lamivudine-resistant HBV variants. HBV DNA polymerase mutations in the YMDD motif (M552I and M552V) and at L528 have been observed in the majority of HBV patients that developed resistance to lamivudine. The F514L mutation also has been found in a few HBV patients that developed resistance to lamivudine. To determine whether adefovir, famciclovir, lobucavir and emtricitabine are cross-resistant with lamivudine, we compared the sensitivity of wild-type and mutant HBV DNA polymerases (F514L, L528M/M552I, and L528M/M552V) to the triphosphate forms of these compounds in enzymatic assays *in vitro*. Our results showed that the F514L, L528M/M552I, and L528M/M552V mutations caused resistance to lamivudine with inhibition constants increased by 6.0-, 15.2- and 25.2-fold, respectively, compared to that of wild type HBV DNA polymerase. However, these mutants remained sensitive to adefovir, lobucavir, and famciclovir with the inhibition constants changing by less than 1.9- 2.3- and 2.5-fold, respectively, compared to wild type. Emtricitabine showed complete cross-resistance with lamivudine, with the inhibition constants increased by 7.5 to 29.7-fold for the F514L, L528M/M552I, and L528M/M552V mutant HBV polymerases. A molecular model of HBV polymerase revealed steric hindrance between the β--methyl branch on the side-chains of amino acids I and V at position 552 in lamivudine-resistant HBV mutants and the sulfur atom in the β-L-oxathiolane ring common to both lamivudine and emtricitabine, thus explaining the overlapping resistance profile of lamivudine and emtricitabine.

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Cellular pharmacology of β-L-thymidine (L-dT, NV-02B) and β-L-2'-deoxycytidine (L-dC, NV-02C) in HepG2 cells and primary rat, monkey and human hepatocytes.

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The cellular pharmacology of L-dT and L-dC, two potent, selective and specific inhibitors of HBV replication, was determined in HepG2 cells and primary human, rat and monkey hepatocytes. L-dT and L-dC (10 μM for 24 hr) were metabolized to 5'-mono-, di- and triphosphate (TP) derivatives with the TP being the predominant intracellular metabolite. Accumulation of the TP increased with extracellular concentration of parent compound. In HepG2 cells the level of TP reached 27.7 ± 12.1 and 72.4 ± 1.8 pmoles/10^6 cells (or μM) for L-dT and L-dC, respectively. No additional metabolites were detected with L-dT. In the L-dC treated cells, mono-, di- and TP forms of β-L-2'-deoxyuridine (L-dU) were also detected. The L-dUTP level reached 18.2 ± 1.1 μM. It is likely that deamination of L-dCMP leads to formation of L-dUMP by deoxycytidylate deaminase, as the parent compound, L-dC, was not a substrate for deoxycytidine deaminase. In primary human hepatocytes, the TP levels for L-dT and L-dC at 24 hr were 16.5 ± 9.9 and 90.1 ± 36.5 μM, respectively. The level of L-dUTP reached 43.5 ± 26.8 μM. Overall, there were no qualitative or quantitative differences in the TP levels of L-dT, L-dC or L-dU in human, rat or monkey hepatocytes. Furthermore, a choline derivative of L-dCDP was detected in human hepatocytes (15.77 ± 1.83 μM at 24 hr) and HepG2 cells. Exposure of HepG2 cells to L-dC + L-dT led to concentrations of the activated metabolites similar to those achieved with either agent alone. The intracellular half-lives of the L-dTTP, L-dCTP and L-dUTP were at least 14 hr. L-dT and L-dC were extensively phosphorylated to the active TP in both human, rat and monkey hepatocytes. L-dCMP was deaminated leading to the formation of L-dUTP, which also exhibited anti-HBV activity.

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The Trimera Mouse System: A Model for HCV Infection and Evaluation of Antiviral Drugs.

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One of the major difficulties in developing therapies against hepatitis C is the lack of in vitro systems or reproducible small animal models for preclinical evaluation of therapeutic candidates. The Trimera mouse system, in which human liver fragments infected with HCV are transplanted into normal strains of mice, provides such a model. The HCV-Trimera model consists of normal mice, lethally irradiated and radioprotected with SCID mouse bone marrow cells, transplanted under the kidney capsule or ear pinna with HCV *ex vivo* infected human liver fragments. Viremia was assessed by RT-PCR followed by dot blot hybridization. Engraftment of viable liver fragments was evaluated by H-E staining and by the presence of HSA mRNA in the grafted tissues. The presence of (-)strand HCV RNA in the grafts indicate viral replication. HCV-RNA in the serum can be detected 8 days after liver transplantation and the levels peak between days 18 and 25, reaching an infection rate of 85%. The HCV-Trimera model was used to evaluate different potential anti-HCV agents. A human monoclonal antibody directed to the HCV envelope protein was able to inhibit HCV infection of human liver tissue as well as to reduce both the percentage of HCV positive animals and the viral load in their sera. Another potential antiviral agent that was positive in a primary IRES assay was also able to reduce viral load in the HCV-Trimera model. Thus, the Trimera mouse model may offer an effective tool for simulating human HCV infection and for evaluating new therapeutic agents.