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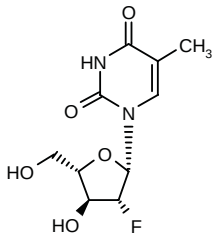
L-FMAU

Anti-HBV
Anti-EBV

Clevudine

1-(2'-Deoxy-2'-fluoro-β-L-arabinofuranosyl)-5-methyluracil
1-(2'-Deoxy-2'-fluoro-β-L-arabinofuranosyl)thymine



$C_{10}H_{13}FN_2O_5$ Mol wt: 260.2240

CAS: 163252-36-6

EN: 217965
EN: 090473 (as β-D-isomer)

Synthesis

L-FMAU (clevudine) can be prepared by two synthetic routes from L-xylose and L-arabinose (Scheme 1):

Previously, it was reported (1) that D-ribose was synthesized from D-xylose, the epimer of D-ribose, via a stereoselective oxidation-reduction procedure, albeit in low yield (2). This strategy was adapted to prepare the L-ribose derivative (V) for the practical synthesis of L-FMAU as well as related nucleosides (3, 4). After pyridinium dichromate (PDC) oxidation, ketone (III) was reduced to an alcohol using $NaBH_4$. It should be mentioned that due to the stereoelectronic effects of the 1,2-*O*-isopropylidene group, the hydride preferentially attacks from the β-face. After a series of reactions, compound (V) was obtained and treated with saturated hydrogen chloride in CH_2Cl_2 at 0 °C followed by the migration of the benzoyl group and treatment with SO_2Cl_2 and imidazole in DMF/CH_2Cl_2 to give a imidazolyl intermediate. The imidazole derivative was fluorinated with a mixture of KHF_2 and 48% HF/H_2O to give 1,3,5-tri-*O*-benzoyl-2-fluoro-α-L-arabinofuranose, which was converted to bromosugar (VI) using 45% HBr/AcOH. Without further purification, the bromosugar was condensed with silylated thymine in anhydrous $CHCl_3$ in refluxing conditions to give protected L-FMAU with high stereoselectivitiy, which was further treated with NH_3/CH_3OH for debenzoylation to give L-FMAU. Due to the availability and high cost of L-xylose, however, we have developed another procedure from L-arabinose. L-Arabinose is a natural sugar with reasonable cost. The anomeric position was protected with a benzyl group in acidic conditions to the pyranose form and the vicinal *cis*-hydroxy groups were protected with an isopropylidene group. In similar conditions as for L-xylose, PDC was used for the oxidation reaction to obtain ketone (IV), which was reduced with $NaBH_4$ in methanol with high stereoselectivity as described for L-xylose. After deprotection of compound (IV) using 4% CF_3CO_2H, the resulting L-ribose was sequentially treated with 1% HCl/MeOH, BzCl/pyridine and H_2SO_4/AcO/AcOH to obtain compound (V). The remaining steps to L-FMAU were the same as described for the L-xylose route. The full account of the synthetic procedure of L-FMAU from L-arabinose will be published elsewhere.

Description

White solid, m.p. 184-5 °C; $[\alpha]_D^{25}$ –111.7° (c 0.23 MeOH); UV (H_2O) λ_{max}: 265.5 (ε 9640) (pH 7), 265.0 (ε 9690) (pH 2), 265.5 (ε 7150) (pH 11); ^{1}H NMR (DMSO-d_6): δ 11.45 (s, NH, D_2O exchangeable), 7.59 (s, H-6), 6.10 (dd, J_{F-H} = 15.4H_3, 1H, H-1'), 5.88 (d, 3'-OH, D_2O exchangeable), 5.13 (t, 5'-OH, D_2O exchangeable), 5.04 (dt, J_{F-H} = 52.8 Hz, 1H, H-2'), 4.22 (dq, J_{F-H} = 18.4 Hz, 1H, H-3'), 3.76 (m, 1H, H-4'), 3.63 (m, 2H, H-5'), 1.78 (s, 3H, CH_3).

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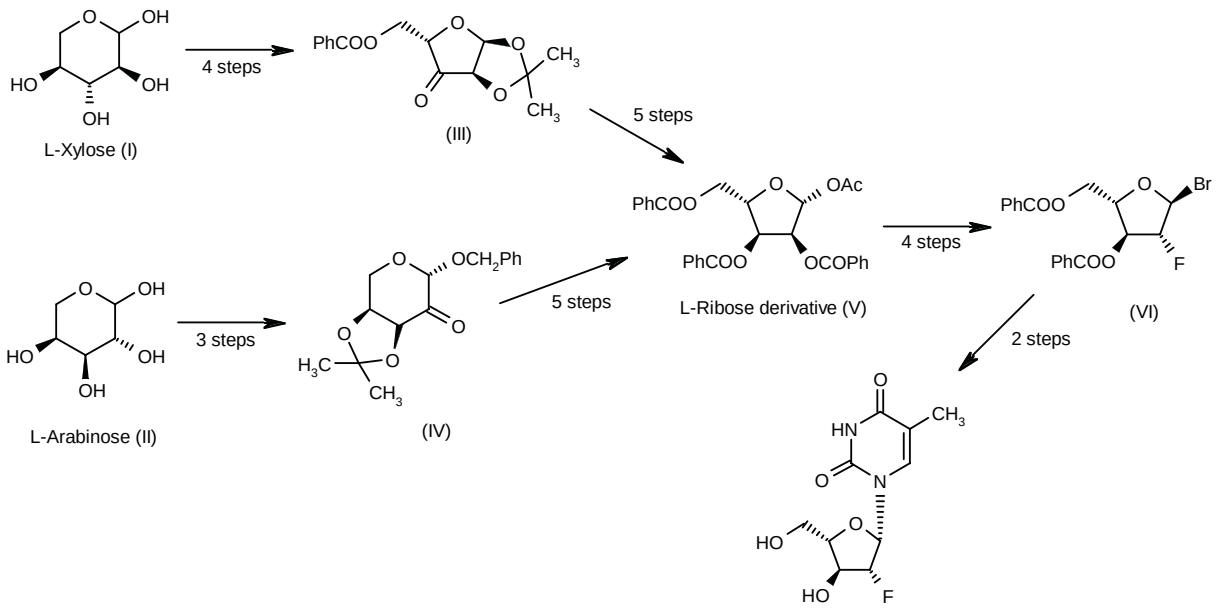
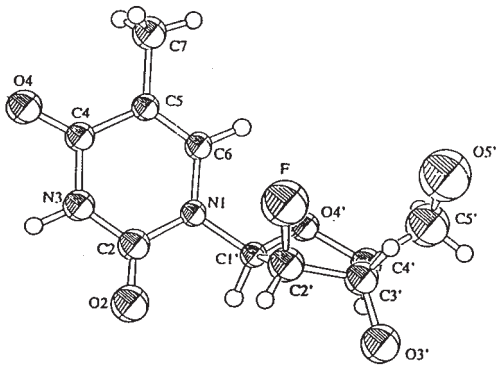
Scheme 1: Synthetic Routes to L-FMAU





ORTEP drawing of L-FMAU

Introduction

Although a number of synthetic as well as natural products have been identified to possess anti-HBV activity *in vitro*, nucleosides are the only class of compounds thus far that appear to be promising for the treatment of chronic HBV infection in humans. In this regard, several nucleoside analogs are currently undergoing preclinical and clinical investigation as potential anti-HBV agents. Recently, our laboratory and others have demonstrated that several nucleosides have potent activity against HBV and HIV. In this article, we describe the properties of 1-(2-fluoro-5-methyl-β-L-arabinofuranosyl)-uracil (L-FMAU) as a potent antiviral agent against HBV and EBV *in vitro* and *in vivo*.

In Vitro Antiviral Activity

The structure-activity relationships of 2'-fluoro-β-L-arabinofuranosyl pyrimidine (3) and purine (4) nucleosides as anti-HBV agents have been established. Among related nucleosides, L-FMAU was found to be the most active compound against HBV *in vitro* (5). Interestingly, it also exhibits antiviral activity against Epstein-Barr virus with an EC_{90} value of approximately 5 μM (6). This is the first example of an L-thymidine analog having potent anti-HBV as well as anti-EBV activity.

In vitro, L-FMAU inhibits HBV viral DNA replication with an EC_{50} value of 0.1 μM in 2.2.15 cells and does not show any significant toxicity up to 200 μM, whereas its D-enantiomer (D-FMAU) was toxic with an IC_{50} value of 50 μM (7). Furthermore, in vitro growth inhibition studies in various cell lines, including MT2, CEM and H1 cell lines, indicated no significant cellular toxicities. Additionally, L-FMAU does not inhibit human bone marrow progenitor cells (5).

The concentration of L-FMAU metabolites in 2.2.15 cells during a period of 24 h was analyzed by HPLC (8).

Rapid conversion of the compound to its phosphorylated forms (L-FMAUMP, L-FMAUDP and L-FMAUTP) could be detected as early as 2 h. With the drug concentrations used, maximal metabolite formations were observed at 8 h. These results suggest that despite the unnatural sugar configuration of L-FMAU, it can be readily metabolized by cells. When the drug was withdrawn, after 24 h treatment, the levels of mono-, di- and triphosphates decreased as a function of time. However, the clearance of the metabolites did not appear to follow first-order kinetics. In the initial phase in drug-free medium, the levels of the metabolites dropped rapidly with about 90% of the triphosphate disappearing in the first 8 h. In the subsequent 16 h, the triphosphate was removed at a lower rate, with 38% of the triphosphate still present at 24 h in comparison to the amount at 8 h. This consistent maintenance of triphosphate levels could be beneficial in the use of L-FMAU as a therapeutic agent.

The uniqueness of L-FMAU among the known anti-HBV agents, such as 3TC, FTC, L-Fd4C, DAPD, lobucavir and BMS-200475, is that it is the first thymidine nucleoside analog shown to have potent anti-HBV activity and it also has unique substrate specificity to all three cellular deoxypyrimidine nucleoside kinases. In order to further investigate whether L-FMAU is phosphorylated by cytosolic dCyd kinase and dThd kinase, the metabolism of L-FMAU was studied in cells that are deficient in these enzymes. L-FMAU was phosphorylated to a lesser extent but still substantially phosphorylated in both dCyd kinase- and dThd kinase-deficient cell lines. dThd and dCyd influenced the degree of L-FMAU phosphorylation in those cell lines. There was a more pronounced inhibition of L-FMAU phosphorylation in cytosolic dCyd kinase-deficient cell lines by dThd than in dCyd kinase-positive cell lines. This is due to the fact that cytosolic dThd kinase is the only major enzyme responsible for its phosphorylation in these cells. Likewise, the phosphorylation of L-FMAU is much less influenced by dCyd in HeLa cells than in dThd kinase-deficient HeLa (Bu) cells. Both dThd and dCyd could suppress L-FMAU phosphorylation to some extent. However, the combination effect of dThd and dCyd was the most effective (9). It should be mentioned that because these kinases lack enantioselectivity, a method for a novel drug design may open up.

Since L-FMAU can be readily metabolized to its phosphorylated forms, the potential for these products to be incorporated into cellular DNA was examined (5). With 5 μM [^{3}H]-L-FMAU treatment for 24 h, there were no significant counts associated with DNA. In contrast to D-FMAU, the acid-insoluble fraction did not show any radioactivity, suggesting that this molecule may not be incorporated. But the positive control, thymidine, was incorporated. Possible explanations for the lack of counts could be either that the triphosphate is not utilized by the cellular polymerase or that the levels of radioactivity under the assay conditions were below the threshold of detection. Thus, the concern of toxicity manifested by D-FMAU during clinical trials due to its incorporation into DNA may not be an issue for L-FMAU.

In addition to its potent anti-HBV activity, L-FMAU also exhibits potent anti-EBV activity. Thus far, no L-nucleoside analogs with anti-EBV activity have been reported in the literature. The EC_{90} value of L-FMAU is as potent as that of DHPG (5.0 μM in H1 cells), whereas the selectivity of L-FMAU is higher than that of DHPG (183 *vs.* 15). However, the amount of L-FMAU nucleotides formed was 3 times larger in EBV producing cells (H1) than in EBV nonproducing cells (L5). The metabolic study of L-FMAU in H1 cells and L5 indicated that it is converted to its mono-, di- and triphosphates. The enzymes involved in this process are still under investigation. Given the differences in the formation of L-FMAUMP in H1 and L5 cells, it is possible that EBV-specified thymidine kinase in H1 cells could utilize L-FMAU as a substrate and could be responsible for the quantitative difference of L-FMAU phosphates formed from uninfected cells. Since L-FMAUMP could also be formed in L5 as well as in other non-EBV containing cells (unpublished results), human enzymes may also be capable of utilizing L-FMAU as a substrate. One interesting feature of L-FMAU metabolism is that the major metabolite is L-FMAUMP. This finding suggests that the intracellular conversion to L-FMAUDP from L-FMAUMP could be the rate-limiting step in the L-FMAU phosphorylation pathway in L5 cells.

To determine whether L-FMAU could be incorporated into cellular or EBV DNA, H1 cells were exposed to equal anti-EBV concentrations of [^{3}H]-L-FMAU (10 μM) or [^{14}C]-D-FMAU (0.2 μM) for 24 h. The nucleic acids were isolated and analyzed using cesium sulfate isopycnic ultracentrifugation. Substantial amounts of [^{14}C]-D-FMAU (higher than 10 pmol/1 million cells) were incorporated into DNA. However, even with 50-fold excess drug treatment, insignificant incorporation of L-FMAU (less than 0.1 pmol/1 million cells) was observed. This finding suggests that the anti-EBV activity of L-FMAU may not be due to its incorporation into EBV DNA. It is also interesting that L-FMAUTP is not a substrate of EBV DNA polymerase (6).

Pharmacokinetics

Initial preclinical pharmacokinetic studies of L-FMAU were conducted in rats (10). Three doses of L-FMAU were administered intravenously (10, 25 and 50 mg/kg) to rats and L-FMAU concentrations in plasma and urine were measured by HPLC. There were no significant differences in the pharmacokinetic parameters among the three doses. Thus, the disposition of L-FMAU was independent of dose over the dosage range of 10-50 mg/kg. Plasma concentrations of L-FMAU declined rapidly with a terminal phase half-life of 1.33 ± 0.45 h (mean ± SD). Total clearance of L-FMAU was moderate, averaging 1.15 ± 0.28 l/h/kg. The fraction of compound excreted unchanged in urine was 0.59 ± 0.13. No glucuronide metabolite was found in urine. The steady-state volume of distribution was 1.12 ± 0.26 l/kg, indicating intracellular distribution of the compound. The fraction of L-FMAU bound to plasma proteins was approximately 15% and

was independent of nucleoside concentration. Bioavailability of L-FMAU following oral administration of 25 mg/kg of L-FMAU averaged 63 ± 13% (11).

Further preclinical pharmacokinetic studies characterized the disposition of L-FMAU in the woodchuck animal model. Woodchucks were given 25 mg/kg L-FMAU intravenously and orally. Following intravenous administration of 25 mg/kg L-FMAU to woodchucks, total clearance averaged 0.13 ± 0.08 l/h/kg and 0.10 ± 0.06 l/h/kg, respectively. Steady-state volume of distribution averaged 0.99 ± 0.17 l/kg, indicating intracellular distribution of the nucleoside. The terminal phase half-life of L-FMAU following intravenous administration averaged 6.2 ± 2.0 h. Absorption of L-FMAU after oral administration was incomplete and bioavailability ranged between 20 and 40%. Slow absorption of L-FMAU resulted in an apparent terminal half-life of 16.2 ± 12.1 h following oral administration. Plasma concentrations of L-FMAU remained above the *in vitro* EC_{50} value of 0.026 μg/ml for hepatitis B virus for 24 h after both intravenous and oral administration of 25 mg/kg of L-FMAU (12).

Toxicity

The effects of L-FMAU on growth in a variety of human cell lines, including HepG2, CEM and H1, indicated its lack of toxicity. L-FMAU was also evaluated in both bone marrow precursor cells (erythroid and granulocyte macrophage) (13) and no significant toxicity was detected up to 100 μM. Since L-FMAU is not an inhibitor or a substrate for DNA polymerase γ, toxicity for mitochondrial function is not expected. In addition, when studies were conducted with isolated human DNA polymerase α, β, δ and γ, L-FMAU failed to be incorporated whereas its D-enantiomer was readily incorporated. These results, especially with human DNA polymerase γ, which is responsible for mitochondrial DNA synthesis, suggest that L-FMAU may not induce mitochondrial toxicity and/or alter its function. The lack of lactic acid production in hepatoma cells repeatedly treated with L-FMAU at high concentrations up to 200 μM in HepG cells suggests that L-FMAU is unlikely to elicit adverse effects similar to those of D-FIAU and D-FMAU (14). Preliminary toxicity studies performed in mice indicated that no apparent toxicity was observed after 30 days of continuous treatment with L-FMAU at 25 mg/kg twice a day (unpublished results).

Preliminary toxicity studies of L-FMAU in comparison to D-FMAU were conducted in woodchucks for 12 weeks at 2 mg/day/kg. The results indicate that there is no change in body weight in L-FMAU-treated animals while there was a marked decline of body weight in D-FMAU-treated woodchucks. These changes were only observed after 7-8 weeks of treatment, which is consistent with the clinical data of D-FIAU (Tennant *et al.*, unpublished results). By the end of the 12-week treatment period, all D-FMAU-treated woodchucks either died or were euthanized because of drug toxicity. The delayed toxicity was associated with mixed microvesicular and macrovesicular steatosis and with lactic acidemia in D-FIAU. No comparable toxicity was observed with L-FMAU-treated or placebo-treated controls (15).

In Vivo Antiviral Activity

In duck animal model, the administration of L-FMAU (40 mg/kg/day) for 5 days by oral route in experimentally infected ducklings (EC_{50} = 0.1 μM) showed a potent inhibition of viral replication (72% compared to control) without any abnormalities, but was followed by a transient rebound of viremia 5 days after drug withdrawal (16), as was also observed with other drugs (17).

Interestingly, a more prolonged protocol with an 8-day administration of L-FMAU in ducklings prevented the rebound of viremia after drug withdrawal and was not associated with an increase of serum lactic acid levels. Southern blot analysis of intracellular viral DNA showed a decrease in the intensity of replicative intermediate at the end of the treatment schedule for L-FMAU-induced culture. At a concentration of 10 μM, L-FMAU induced a dramatic inhibition of viral DNA synthesis. However, viral CCC DNA was still detected at the end of therapy. Although DHBV POL gene mutants were not selected during short-term administration of L-FMAU, it remains to be determined whether this phenomenon could be observed during long-term therapy. After drug withdrawal, the persistence of viral DNA and proteins was associated with low level viremia under the limit of detection of dot blot assay, which may suggest either a decrease in viral particle secretion from infected hepatocytes or an enhanced clearance of viral particles from the serum. No significant signs of cytotoxicity were observed during the daily microscopic examination of cultured cells. Testing of the culture supernatant for lactic acid showed no increase in lactic acid levels during cell culture, regardless of the therapeutic protocol (18).

The efficacy studies of L-FMAU were also conducted in woodchucks chronically infected with WHV: L-FMAU was given orally to 4 chronic woodchuck hepatitis virus (WHV) carriers at a daily dose of 10 mg/kg for 12 weeks (19). There was prompt and significant inhibition of viral replication compared to placebo-treated controls. Within 7 days, the plasma WHV DNA of drug-treated woodchucks decreased more than 1000-fold during treatment and was undetectable by conventional dot blot hybridization for at least 22 weeks post-treatment. The $t_{1/2}$ of plasma WHV DNA during the initial phase of drug treatment averaged 17 ± 1 h (range 16-19 h). A significant decrease in mean hepatic WHV DNA replicative intermediate (RI) also was observed. After 12 weeks of L-FMAU treatment, mean hepatic WHV DNA RI decreased more than 100-fold. Episomal WHV DNA (3.2 Kb WHV DNA), a minor component of intracellular WHV DNA, also decreased moderately at the end of treatment. Immunohistochemical studies of hepatic WHc expression demonstrated significant reduction at the end of treatment in 2 woodchucks, while in 2 others, only moderate reduction of WHc

expression in the liver was observed. At 4 weeks and 12 weeks post-treatment, there was a further reduction in WHV DNA RI. WhcAg expression decreased in all 4 woodchucks at 4 weeks post-treatment, and WhcAg staining was considered negative 12 weeks following the end of treatment. Decreases in body weight observed in L-FMAU-treated woodchucks were similar to those of control, and no other physical or biochemical evidence of drug-related toxicity was observed. Furthermore, no significant rebound was observed up to 18 weeks after discontinuation of L-FMAU and 3 of 4 animals remained undetectable by the dot blot method up to 50 weeks. However, 1 animal experienced several flares beyond 18 weeks of drug discontinuation.According to the 4-week dose-escalation study, a rapid and profound suppression in serum WHV DNA was observed at L-FMAU doses ranging from 0.3-10 mg/kg orally once daily. The reduction in viral DNA was most marked at the 10 mg/kg dose and least marked at the lowest (0.3 mg/kg) dose. By comparison, WHV DNA levels were approximately 400-fold less after 3 weeks of treatment with L-FMAU at 10 mg/kg orally daily. L-FMAU at doses of 1 mg/kg and 3 mg/kg has resulted in 70-fold and 125-fold reductions of mean WHV DNA, respectively, when compared to pretreatment values. Viral recrudescence was observed following drug withdrawal within 4 weeks in those woodchucks receiving L-FMAU at 0.3 mg/kg and 1 mg/kg daily (20).

Summary

L-FMAU is a potent anti-HBV nucleoside in 2.2.15 cells with a favorable toxicity profile in various cell lines *in vitro*. L-FMAU is phosphorylated to its monophosphate by cellular dCyd kinase, dThd kinase and mitochondrial dPyrd kinase. But cytosolic dThd kinase is the major enzyme responsible for its phosphorylation in these cells. The monophosphate is further phosphorylated to di- and triphosphate. The triphosphate inhibits HBV and EBV DNA polymerases, which appears to be the mechanism of L-FMAU as an antiviral agent. However, L-FMAU is not incorporated to HBV/EBV DNA. Interestingly, the major metabolite of L-FMAU in HBV containing cells is L-FMAUTP in comparison to LFMAUMP in EBV containing cells. Futhermore, the triphosphate does not interact with the cellular DNA polymerase α, β, δ and ε. This may explain the low toxicity of L-FMAU *in vitro* and *in vivo*. Pharmacokinetic studies in rats and woodchucks indicated that L-FMAU disposition is typical of nucleoside analogs. The compound is orally bioavailable in rats and woodchucks. Preliminary toxicity studies conducted in mice and woodchucks suggest that L-FMAU does not exhibit any marked toxicity in these animal models while D-FMAU showed significant toxicity. *In vivo* efficacy studies conducted in chronically infected woodchucks at 10 mg/day/kg for 50 weeks indicated that L-FMAU is a potent anti-HBV agent and no significant viral rebound was observed even after discontinuation of the drug. Therefore, potent *in vitro* and *in vivo* efficacy, favorable toxicity in animal models and various cell lines, as well as its unique structure, metabolism and mechanism make L-FMAU a potential clinical candidate as an anti-HBV agent.

Acknowledgements

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Manufacturers

Bukwang Pharm. Co. (KR) and Triangle Pharm. Co. (US).

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