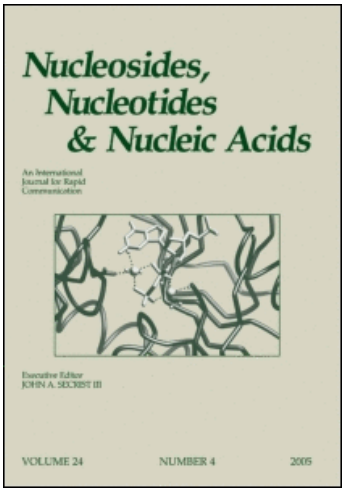

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In Vitro and In Vivo Metabolism and Pharmacokinetics of *bis* [(T-Butyl)-S-acyl-2-thioethyl]-β-L-2',3'-dideoxy-5-fluorocytidine Monophosphate

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IN VITRO AND IN VIVO METABOLISM AND PHARMACOKINETICS OF BIS [(T-BUTYL)-S-ACYL-2-THIOETHYL]-ß-L-2',3'-DIDEOXY-5-FLUOROCYTIDINE MONOPHOSPHATE

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Dedicated to the memory of Dr. Gertrude B. Elion.

ABSTRACT: Exposure to 10 &M L-FddCMP-bisSATE led to formation of intracellular L-FddCTP levels of 410.1± 46.2 and 242.1 ± 13.2 pmol/10^6 cells in unstimulated and PHAstimulated PBM cells, respectively; whereas, exposure of cells to the parent nucleoside, L-FddC, generated 5 - 10-fold less L-FddCTP. In Hep-G2 cells and EGF/HGF stimulated and unstimulated primary cultured hepatocytes, the active metabolite reached 113 ± 29, 23.9 ± 15.6, and 20.6 ± 10.5 pmol/10^6 cells. Three other metabolites, L-FddCMP-monoSATE, L-FddCMP-SH, and M I, were detected intracellularly and extracellularly in all cell types examined. Intravenous administered dose of 3 mg/kg L-FddCMP-bisSATE to rhesus monkeys resulted in plasma concentration levels of 2.06 ± 1.00 and 0.39 ± 0.15 &M of L-FddCMP-monoSATE and L-FddC, respectively, while the prodrug was completely cleared metabolically within 15 min. Following oral administration of an equivalent dose, the absolute oral bioavailability of L-FddC derived from L-FddCMP-bisSATE administration was 65%.

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INTRODUCTION

Nucleoside analogs have consistently been a main component in anti-human immunodeficiency virus (HIV) therapeutic regimens which often are comprised of a minimum of three agents, one or two of which usually are nucleoside analogs. Highly active anti-retroviral therapy (HAART) with various combinations of HIV reverse transcriptase (RT) inhibitors, nonnucleoside reverse transcriptase inhibitors (NNRTI), and HIV protease inhibitors has proven to be effective in reducing serum viral load to below detection limits and substantially increasing CD4+ cells within the immune system. However, low levels of viral particles persist in various tissues such as lymph nodes and various cell types such as nonactivated lymphocytes. Total eradication of HIV from host cells and tissues by effective antiviral agents must address this critical issue. This task is further complicated by the complex issue of emerging resistant HIV populations that can lead to the eventual failure of HAART. These obstacles warrant the development of effective antiviral agents that possess potent and selective antiviral activity and good oral bioavailability.

The nucleoside analogs that target HIV-RT are not intrinsically active themselves. They must be sequentially phosphorylated by host cellular kinases to their respective 5'-triphosphate (TP) derivatives. The nucleoside triphosphate's mechanism of antiviral activity includes direct competitive inhibition of the viral polymerase and incorporation into viral DNA leading to chain termination. The initial phosphorylating enzyme for dideoxynucleoside (ddN) analogs is often the most discriminating step in the three step phosphorylation pathway. Furthermore, this initial phosphorylating enzyme can be cell cycle regulated. These two issues have hindered the development of some possible effective antiviral agents whose 5'-TP derivatives are extremely potent inhibitors of HIV-RT. For example, the 5'-TP derivative of ß-D-2'3'-dideoxyuridine (ddU) is an extremely potent inhibitor of HIV-RT with a K_i of 0.05 &M; however, ddU is an extremely poor inhibitor of HIV *in vitro*, and this has been attributed to the lack of sufficient generation of ddUMP, intracellularly.[1] Kinetic studies demonstrated that ddU is a poor substrate for enzymes that phosphorylate uridine nucleoside analogs.[2] To circumvent this obstacle, direct intracellular delivery of the nucleoside monophosphate has been successfully implemented, *in vitro*.[2,3] The main impediment to delivering the nucleoside monophosphate is its low lipophilicity due to the two negative charges associated with the 5'-phosphate group. In order to mask this dianionic charge, phosphoester linked biolabile groups have been attached to the 5'-phosphate group.

Various strategies with different masking groups have been investigated and have been demonstrated to exhibit differences in decomposition kinetics, generated intermediates, and intracellular and extracellular stability.[4] S-acyl-2-thioethyl (SATE) pronucleotides for ddUMP, ddAMP, D4TMP, AZTMP, PMEA, and acyclovir monophosphate have been synthesized and proven to be potent anti-HIV and anti-HBV agents, *in vitro*.[5-10] Other strategies links two S-(2-hydroxyethylsufidyl)-2-thioethyl (DTE) leaving groups, or two pivaloyloxymethyl (POM) groups, or two isopropyloxycarbonyloxymethyl (POC) groups to mask the dianionic charge on nucleoside monophosphates.[11-13] Another chemical design strategy incorporates a carbocyclic moiety bridging the two negative charges on the phosphate.[14-17] This approach uniquely relied on P-450 enzymes to liberate the monophosphate. However, this approach by Farquhar et. al.

was unsuccessful due to intracellular stability of the prodrug. This demonstrates the importance of the chemical structure's inherent susceptibility to enzymatic decomposition. Each of the above chemical designs have advantages as well as disadvantages when considering extracellular and intracellular decomposition kinetics, efficacy, toxicity, oral bioavailability, cell specificity, pharmacokinetics, tissue distribution, and protein binding.

Previous studies have shown L-FddC to be a potent anti-HIV and anti-hepatitis B virus (HBV) agent *in vitro* at nanomolar concentrations in contrast to its corresponding ß-D enantiomer, ß-D-2',3'-dideoxy-5-fluorocytidine (D-FddC).[18,19] The greater potency of L-FddC has been attributed to its extensive phosphorylation to its respective 5'-triphosphate and the extended intracellular half-life of L-FddCTP.[20] Upon intravenous bolus administration to rhesus monkeys, L-FddC recovery in the urine was only 47%.[21] Previously, an increase in the oral bioavailability of the acyclic monophosphate, PMEA, has been achieved by administering its prodrug, bis-POM-PMEA.[22,23] In order to attempt an improvement of the oral bioavalability of L-FddC, the bis-SATE derivative of L-FddCMP was synthesized and its distribution and pharmacokinetics were assessed in rhesus monkeys. Furthermore we analyzed the intracellular pharmacology of bis[(t-butyl)SATE]-L-FddCMP (L-FddCMP-SATE) in Hep-G2 cells, hepatocyte growth factor (HGF) and epidermal growth factor (EGF) stimulated and unstimulated primary cultured human hepatocytes, phytohemagluttanin (PHA) stimulated peripheral blood mononuclear (PBM) cells, and nonstimulated PBM cells to examine the intracellular anabolic effect of bypassing the initial phosphorylating enzyme in dividing and quiescent cells with SATE nucleotide analogs.

MATERIALS AND METHODS

Compounds

The stereoselective synthesis of L-FddC from L-xylose has been reported elsewhere.[24] L-FddCMP-bisSATE was synthesized by using a P(III) approach as previously described.[5] Briefly, bis (*tert* - butyl-S-acetyl-2-thioethyl)-diisopropyl aminophosphite diester was directly condensed with ß-L-FddC in the presence of tetrazole followed by *in situ* oxidation with 3-chloroperoxybenzoic acid. The corresponding product was characterized by nuclear magnetic resonance (^{1}H, ^{31}P), fast atom bombardment mass spectroscopy, HPLC, and UV spectroscopy, and it was consistent with the chemical structure. [^{3}H]-Bis[(t-butyl)SATE-ß-L-FddCMP (22.8 Ci/mmol) and [6-^{3}H]-ß-L-FddC (2.5 Ci/mmol) were purchased from Moravek Biochemical (Brea, CA), and their purity was assessed to be greater than 99% by HPLC. All chemicals and reagents used were of the highest grade available.

Cell Culture, Exposure, and Harvest

Three different cell types (Hep-G2, PBM cells, and primary cultured human hepatocytes) were used to assess the intracellular pharmacology of L-FddCMP-bisSATE. Due to the limited availability of [^{3}H]-L-FddC, only PHA-stimulated PBM cells were exposed to 10 µM L-FddC for 48 hours. Culture and harvest condition for Hep-G2 cells were identical to those previously employed.[20] Primary cultured human hepatocytes were obtained either through the UAB Liver Center or from Clonetics Corporation (San Diego, CA). Hepatocytes were isolated from liver transplant donors through a two-step collagenase perfusion procedure and seeded as previously

reported.[25] Primary cultured human hepatocytes were stimulated with 100 ng/mL EGF and 10 ng/mL HGF for 72 hours in Leibovitz medium supplemented with 2 mM glutamine, 50 units/mL penicillin, 50 μg/mL streptomycin, and 0.1 units/mL insulin. PBM cells were obtained from whole blood by single-step Ficoll-Histopaque discontinuous gradient centrifugation. The cell population was divided into two groups and cultured in RPMI 1640 medium supplemented with 10% FBS, 1000 U/mL penicillin, 1000 μg/mL streptomycin, and 2 mM glutamine. Each cell line was exposed to 10 μM L-FddCMP-bisSATE in their respective culture medium without FBS. After 1, 4, 8, and 24 hours, the L-FddCMP-bisSATE exposed Hep-G2 cells and PBM cells were collected by centrifugation at 350x*g* or 600x*g*, respectively. The extracellular medium was collected. The cells were then washed three times with ice-cold PBS and extracted overnight with 60% methanol. Intracellular extracts were dried under a gentle stream of nitrogen and reconstituted in 160 μL of deionized water. After 1, 4, 8, and 24 hours, the medium was collected from the adherent primary cultured human hepatocytes exposed to L-FddCMP-bisSATE, and the monolayers were washed three times with ice-cold PBS. Cells were scrapped off the plates in 60% methanol and extracted overnight. Extracts were then dried under nitrogen and reconstituted in 160 μL of deionized water. All intracell and extracell samples were kept at -70° C until HPLC analysis.

Animal Study Design

Rhesus monkeys (*Macaca mulatta*) were used for the *in vivo* metabolism and pharmacokinetic studies. These animals were maintained at the Yerkes Regional Primate Research Center at Emory University in accordance with guidelines established by the Animal Welfare Act and the NIH *Guide for the Care and Use of Laboratory Animals*. The Yerkes Center is fully accredited by the American Association for Accommodation of Laboratory Animal Care. Three young adult female rhesus monkeys weighing between 5.2 and 5.9 kg were used for intravenous and oral administration of L-FddCMP-bisSATE in this study. Monkeys were fasted for 12 h prior to dose administration, and water was made available *ad libitum* throughout the fasting and post anesthesia period. For intravenous dosing, three monkeys (RNa5, RSa5, and RUa5) received a 5 mg per kilogram body weight bolus dose of L-FddCMP-bisSATE with a 250 μCi [^{3}H]-L-FddCMP-bisSATE tracer dissolved in 50%/50% (v:v) sterile PBS (pH 7.4) and ethanol mixture. Following a 2-month washout period, the same animals were administered an equivalent oral dose by nasogastric intubation with thorough flushing of the administration tube. There was no substantial weight fluctuation of the animals between the intravenous and oral dose administrations.

Two milliliters of blood was collected in a clot tube prior to and at 0.25, 0.5, 1, 2, 4, 6, 8, 24, and 48 hours after dose administration. A catheter was inserted into the bladder for urine collection for the first 8 hours. Afterwards, urine was collected from the pan beneath the cage at 24 hours and every 24 hours until 14 days after dose administration. Monkeys were initially anesthetized with a combination of Telazol and Ketamine with supplemental doses administered periodically as needed. Anesthesia was halted after eight hours, and the bladder catheter was removed. Animals were briefly anesthetized for blood collection at the 24 and 48 h. No feces samples were collected. Feces excreted after 24 h were monitored for radioactivity by wipe test until levels were below baseline. Plasma and urine samples were then frozen at -70° C until

analysis. Due to radioactivity levels in conjunction with plasma protein binding, plasma samples were injected directly onto the HPLC system. Urine samples were filtered through a 0.2 μm Acrodisc filter and injected onto the HPLC system.

Pharmacokinetic Analysis

Pharmacokinetic parameters for L-FddCMP-bisSATE catabolites were estimated by a model-independent approach using SIPHAR/Base software.[26] Exponential curves were generated using an initial peeling algorithm and refined using a weighted $(1/y_{(calc.)}^2)$ least-squares computational algorithm with extrapolation to infinity based on the terminal slope of the elimination phase. Half-life ($T_{1/2}$) was estimated as 0.693/K, where K is the slope of the corresponding distribution or elimination phase. The maximum concentration of each metabolite in plasma (C_{Max}) was obtained from observed data points, and T_{Max} was the time to C_{Max}. When C_{Max} occurred after the first time point, exponential curves were generated using an extravascular bolus model, and these curves were not forced through the origin. The lag-time was based on the intersection of the two residual lines obtained using a peeling algorithm. Specific time-interval area under the concentration versus time curve (AUC) for L-FddCMP-bisSATE metabolites was obtained using the trapezoidal rule. Renal clearance (CL_R) was calculated as the quotient of the amount of the metabolite excreted in the urine during a defined time interval and the corresponding time interval plasma AUC. Oral bioavailability of L-FddC, liberated from the prodrug L-FddCMP-bisSATE, was calculated as the quotient of the AUC of L-FddC derived from orally administered L-FddCMP-bisSATE and the AUC of L-FddC derived from intravenously administered L-FddC.

Analytical HPLC Methodology

Cell extracts from stimulated PBM cells exposed to L-FddC were analyzed by a previously published ion-exchange HPLC method and retention times were identical to those previously reported.[20] Under these conditions, the limit of detection was 0.1 pmol/10^6 cells. Intracellular, extracellular, monkey plasma, and urine samples from exposure to and administration of L-FddCMP-bisSATE were analyzed by ion-pairing HPLC on a Hewlett-Packard model 1090 equipped with an automatic injector and a fixed-wavelength UV spectrophotometer. The stationary phase was a 25 cm Hypersil C-18, 5 μm, octadecylsilane column from Jones Chromatography (Lakewood, CO) preceded in series by a 5 μm RP-18 LiChrospher HPLC guard column from EM Science (Gibbstown, NJ). The mobile phase consisted of 0.1 M triethylammonium bicarbonate (TEAB), pH = 7.4, and acetonitrile. Elution was conducted at 1 mL/min with a multiple-step linear gradient beginning with 2% acetonitrile and sequentially increasing to 3% at 10 minutes. This percentage is maintained until 20 minutes and then increased to 6% at 30 minutes, 25% at 40 minutes, and 80% at 55 minutes until the end of the run. Eluted radioactivity was monitored by an online 500TR Radiomatic FLO-ONE radiochromatograph from Packard Instrument Company (Meriden, CT). Under these conditions the retention times of the parent drug, L-FddCMP-bisSATE, its metabolites, L-FddCMP-monoSATE, L-FddCMP-SH, L-FddC, L-FddCMP, L-FddCDP, L-FddCDP-choline, and L-FddCTP were 54, 45, 39, 16, 8, 12, 6, and 14 minutes, respectively, and the limit of detection

was 0.1 pmol/10^6 cells. Additionally, an unidentified metabolite, designated as MI, eluted at 37 minutes.

Plasma Protein Binding and Stability in Whole Blood

Plasma protein binding was assessed in Centrifree micropartition devices from Amicon, Inc. that have a size exclusion limit of 30,000 daltons (Beverly, MA). 200 µL of human plasma was spiked with 10 µM L-FddCMP-bisSATE and 1 µCi of [^{3}H]-L-FddCMP-bisSATE. Nonspecific binding was accounted for by spiking PBS with identical L-FddCMP-bisSATE concentration and radioactivity. Samples were centrifuged at 2000 x *g* for two hours in a Beckman JA-20.1 fixed-angle rotor. Total radioactivity was assessed in the sample before centrifugation and in the ultrafiltrate and retentate after centrifugation. Additionally, protein binding in rhesus monkeys was assessed by determining the AUC of the protein bound entity's concentration versus time curve. Stability of L-FddCMP-bisSATE in whole blood was determined by spiking whole blood with a 10 µM concentration and incubating aliquots for 5, 10, and 15 minutes at 37° C.

RESULTS

Stimulated and Unstimulated Primary Cultured Human Hepatocytes

L-FddCMP-bisSATE was rapidly taken up in both stimulated and unstimulated hepatocytes and rapidly decomposed to its corresponding monoSATE derivative. Intracellular and extracellular concentrations of L-FddCMP-bisSATE, metabolites, and its 5'-phosphorylated derivatives are presented in Table 1. The intracellular decomposition of L-FddCMP-bisSATE to L-FddCMP and the subsequent anabolism to its respective 5'phosphorylated derivatives is presented in Figure 1. Livers from five donors contributed to the high standard deviation and demonstrated significant interindividual variations in the metabolism of L-FddCMP-bisSATE. Low intracellular and extracellular L-FddCMP-bisSATE levels were detected in both stimulated and unstimulated hepatocytes at 1 hour and were below detection by 4 hours. Rapid intracellular accumulation and extracellular appearance of L-FddCMP-monoSATE was detected by 1 hour. Intracellular L-FddCMP-monoSATE levels of 59.2 ± 61.4 pmol/10^6 cells in stimulated hepatocytes and 5.8 ± 3.9 pmol/10^6 cells in unstimulated hepatocytes were detected at 1 hour and gradually declined over the 24 hour exposure. Extracellular L-FddCMP-monoSATE levels remained consistent at approximately 5 µM throughout the 24 hour exposure in both stimulated and unstimulated cells. At one hour, the thiol derivative, L-FddCMP-SH, achieved a peak intracellular concentration of 15.4 ± 9.5 and 5.9 ± 7.1 pmol/10^6 cells in stimulated and unstimulated hepatocytes, respectively. L-FddCMP-SH concentration declined to a level below detection at 24 hours in the stimulated hepatocytes and by 8 hours in the unstimulated hepatocytes. Similar trends were observed with MI, but its level declined below detection limits at 24 hours only in unstimulated hepatocytes. Rapid delivery of L-FddCMP was achieved within one hour with 34.8 ± 21.4 and 30.8 ± 9.8 pmol/10^6 cells measured at 1 hour in stimulated and unstimulated cells, respectively. High intracellular ß-L-FddCMP levels provided adequate substrate to generate peak intracellular ß-L-FddCTP levels of 23.9 ± 15.6 and 20.6 ± 10.5 pmol/10^6 cells by 4 hours in both stimulated and unstimulated cells, respectively.

Table 1. Intracellular accumulation of 10μM L-FddCMP-bisSATE metabolites in unstimulated (U) hepatocytes and hepatocytes stimulated (S) with 100 ng / mL EGF and 10 ng / mL HGF for 72 hrs.

Compound	Status	Intracellular Concentration (pmol / 10^6 cells)			
		Time (hr)			
		1	4	8	24
L-FddCMP-bisSATE	S	6.3 ± 6.4[a]	ND[b]	ND	ND
	U	0.3 ± 0.3	ND	ND	ND
L-FddCMP-monoSATE	S	59.2 ± 61.4	21.7 ±20.9	1.3 ±1.5	1.9 ± 3.2
	U	5.8 ± 3.9	2.0 ± 2.1	1.4 ± 0.7	0.6 ± 0.3
L-FddCMP-SH	S	15.4 ± 9.5	6.6 ± 6.2	2.0 ± 3.4	ND
	U	5.9 ± 7.1	0.7 ± 1.4	ND	ND
MI	S	27.2 ± 24.1	10.7 ± 8.9	2.3 ± 2.0	0.4 ± 0.7
	U	8.5 ± 14.9	2.2 ± 4.7	0.5 ± 0.7	ND
L-FddC	S	6.8 ± 15.3	6.8 ± 8.1	8.2 ± 17.9	3.2 ± 5.3
	U	1.4 ± 1.3	2.4 ± 2.4	1.3 ± 1.1	2.1 ± 1.9
L-FddCMP	S	34.8 ± 21.4	32.4 ± 24.2	16.1 ± 12.9	7.4 ± 7.6
	U	30.8 ± 9.8	17.0 ± 12.8	14.4 ± 11.3	11.8 ± 9.3
L-FddCDP-choline	S	0.5 ± 0.4	0.5 ± 0.5	0.8 ± 0.8	1.0 ± 1.5
	U	1.1 ± 0.6	0.7 ± 0.9	0.4 ± 0.7	0.8 ± 1.2
L-FddCDP	S	12.5 ± 3.7	33.2 ± 27.8	22.5 ± 19.7	11.2 ± 8.0
	U	17.0 ± 6.6	10.5 ± 4.9	8.2 ± 1.7	5.7 ± 1.6
L-FddCTP	S	8.3 ± 5.3	23.9 ± 15.6	23.3 ± 21.1	10.7 ± 6.4
	U	15.7 ± 9.4	20.6 ± 10.5	16.8 ± 5.8	12.8 ± 10.8

a: Data are mean ± S.D. of cells obtained from five different donors.
b: ND = not detected.

Hep-G2 Cells

Intracellular accumulation and metabolism of 10 μM L-FddCMP-bisSATE was also detected in the well characterized hepatoblastoma cell line Hep-G2. These results are presented in Table 2. Intracellular L-FddCMP-bisSATE and L-FddCMP-monoSATE were below the level of detection before 1 hour, and L-FddCMP-bisSATE was not detected in the extracellular medium at 1 hour. However, the monoSATE derivative was detected extracellularly at 1.7 ± 0.2

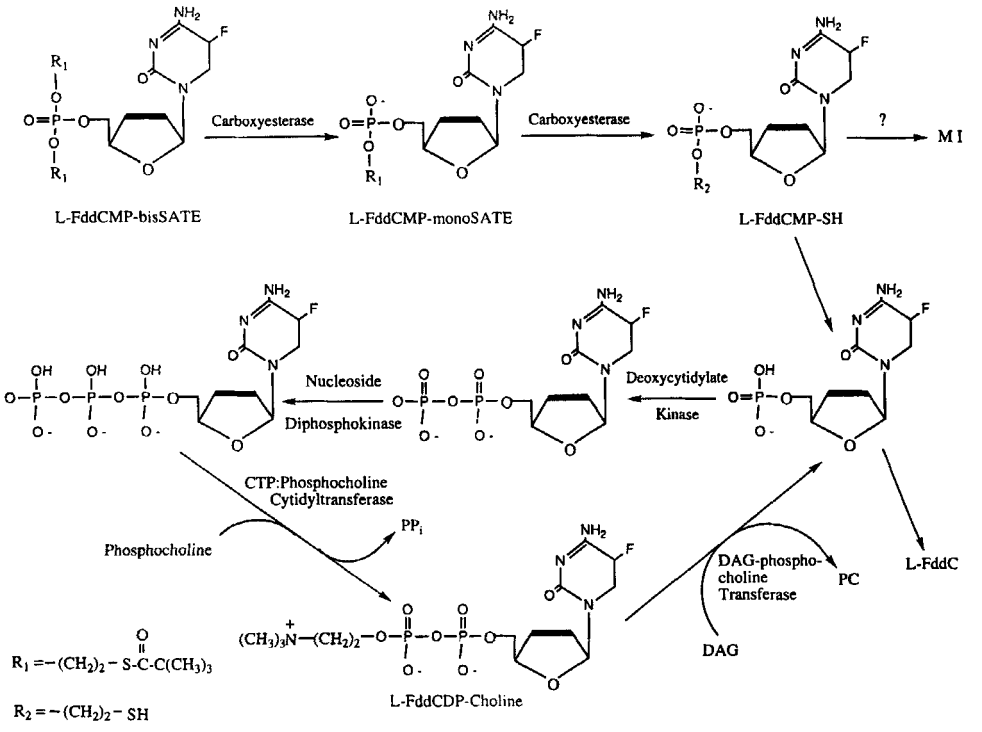

Figure 1. Metabolic pathway for the decomposition and anabolism of L-FddCMP-bisSATE. Abbreviations: DAG, 2,3-diacylglycerol; PC, phospatidylcholine; PP_i, inorganic pyrrophosphate.

Table 2. Intracellular accumulation of 10μM L-FddCMP-bisSATE metabolites in Hep-G2 cells over specified time periods.

Compound	Intracellular Concentration (pmol / 10^6 cells)			
	Time (hr)			
	1	4	8	24
L-FddCMP-bisSATE	ND[a]	ND	ND	ND
L-FddCMP-monoSATE	ND	ND	ND	ND
L-FddCMP-SH	30 ± 1	ND	ND	ND
MI	7 ± 2	3 ± 2	ND	ND
L-FddC	6 ± 1	2 ± 1	2 ± 1	2 ± 1
L-FddCMP	141 ± 17	49 ± 4	34 ± 7	37 ± 2
L-FddCDP-choline	5 ± 1	3 ± 1	8 ± 1	51 ± 3
L-FddCDP	58 ± 5	67 ± 11	55 ± 1	40 ± 1
L-FddCTP	69 ± 12	113 ± 29	64 ± 5	32 ± 1

a: ND = not detected.

μM by 1 hour and the extracellular concentration remained consistent for 24 hours. L-FddCMP-SH at a concentration of 30 ± 1 pmol/10^6 cells was detected intracellularly by 1 hour and declined to levels below detection by 4 hours. Initial intracellular concentration of the unidentified metabolite, MI, of 7 ± 2 pmol/10^6 cells was detected at 1 hour and declined to levels below detection by 8 hours. Extracellular MI was below detection until 8 hours when a low level of 0.1 ± 0.1 μM was detected and remained consistent for the duration of the 24 hour exposure. Rapid intracellular liberation of L-FddCMP occurred with 141 ± 17 pmol/10^6 cells detected at 1 hour. This level substantially declined to 34 ± 7 pmol/10^6 cells by 8 hours with a concomitant peak in intracellular L-FddCTP of 113 ± 29 pmol/10^6 cells at 4 hours. 6 ± 1 pmol/10^6 cells of L-FddC was detected at 1 hour and declined to 2 ± 1 pmol/10^6 cells by 4 hours. During 5'-phosphorylated derivatives intracellular accumulation, low intracellular levels of the diphosphocholine derivative, L-FddCDP-choline, were detected at 1 and 4 hours and exponentially increased to 51 ± 3 pmol/10^6 cells by 24 hours. The intracellular decomposition of L-FddCMP-bisSATE to L-FddCMP and the subsequent anabolism to its respective 5'phosphorylated derivatives is presented in Figure 1.

Stimulated and Unstimulated PBM Cells

Human PHA-stimulated PBM cells were exposed to 10 μM L-FddC for 48 hours. L-FddCTP reached a peak concentration of 50.1 ± 10.7 pmol/10^6 cells at 48 hours with no apparent cytotoxicity. L-FddCMP and L-FddCDP gradually increased throughout the exposure to a peak concentration of 9.5 ± 1.6 and 60.1 ± 11.5 pmol/10^6 cells, respectively, at 48 hours. L-FddCDP-choline achieved a concentration of 4.0 ± 1.3 pmol/10^6 cells at 48 hours. These results are presented in Table 3. Human PHA-stimulated and unstimulated PBM cells were also exposed to 10 μM L-FddCMP-bisSATE for 24 hours. Intracellular and extracellular metabolite levels were quantitated, and these results are presented in Table 4. L-FddCMP-bisSATE was rapidly taken up and converted to L-FddCMP-monoSATE by one hour with a L-FddCMP-monoSATE intracellular concentration of 2.8 ± 0.6 and 2.0 ± 0.2 pmol/10^6 cells in stimulated and unstimulated PBM cells, respectively. These levels declined below detection by 24 hours. Simultaneously, extracellular L-FddCMP-bisSATE concentration declined to 0.05 ± 0.01 and 0.15 ± 0.03 μM at 24 hours, and extracellular L-FddCMP-monoSATE concentration increased with time to 1.75 ± 0.24 and 1.37 ± 0.05 μM at 24 hours in stimulated and unstimulated PBM cells, respectively. L-FddCMP-SH and MI were detected intracellularly by 1 hour at 19.4 ± 11.2 and 47.5 ± 0.7 pmol/10^6 cells, respectively in stimulated PBM cells. Intracellular L-FddCMP-SH level remained approximately consistent through 8 hours, and declined below detection at 24 hours while intracellular MI concentration declined with time to a level below detection by 24 hours. Extracellular L-FddCMP-SH and MI were not detected at 1hour, but their concentration increased with time to 0.09 ± 0.04 and 0.05 ± 0.01 μM at 24 hours, respectively. Rapid intracellular liberation of L-FddCMP occurred with 66.1 ± 22.5 pmol/10^6 cells detected intracellularly at 1hour. Intracellular L-FddCMP increased to 89.8 ± 6.6 pmol/10^6 cells at 4 hours, and declined afterwards to 11.8 ± 9.3 pmol/10^6 cells at 24 hours. Simultaneously, intracellular L-FddCTP level increased to a peak concentration of 242.1 ± 13.2 pmol/10^6 cells at 8 hours. A maximum intracellular L-FddCDP-choline concentration of 4.8 ± 0.3 pmol/10^6 cells was also detected at 8 hours.

Table 3. Intracellular accumulation of 10μM L-FddC anabolites in human peripheral blood mononuclear cells stimulated with 0.1% phytohemagluttanin for 72 hours.

Compound	Intracell Concentration (pmol / 10^6 cells)				
	Time (hr)				
	2	4	8	24	48
L-FddCMP	0.5 ± 0.1[a]	1.1 ± 0.1	3.4 ± 2.3	7.2 ± 1.4	9.5 ± 1.6
L-FddCDP-choline	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	1.8 ± 0.1	4.0 ± 1.3
L-FddCDP	2.9 ± 0.2	7.1 ± 0.9	13.8 ± 1.8	42.9 ± 5.2	60.1 ± 11.5
L-FddCTP	2.2 ± 0.5	5.9 ± 2.8	10.0 ± 5.0	34.6 ± 10.7	50.1 ± 10.7

a: Data are mean ± S.D. of cells from one donor performed in triplicate.

In comparison to PHA-stimulated PBM cells, approximately equal intracellular levels of L-FddCMP-bisSATE and the monoSATE derivative were detected until 8 hours in unstimulated cells. However, both intracellular bis- and monoSATE concentrations were below detection limits at 24 hours in stimulated cells. In contrast to stimulated cells, intracellular L-FddCMP-SH and MI levels were still detected at 24 hours with levels of 3.9 ± 2.2 and 14.8 ± 1.3 pmol/10^6 cells, respectively, in stimulated cells. Significantly greater levels of intracellular 5'-phosphorylated derivatives and the 5'-diphosphocholine derivative of L-FddC were achieved in unstimulated PBM cells. Maximum L-FddCTP concentration (410.1 ± 46.2 pmol/10^6 cells) and L-FddCDP-choline concentration (23.9 ± 0.7 pmol/10^6 cells) was achieved at 24 hours. These results are presented in Table 4.

Stability of L-FddCMP-bisSATE in Whole Blood and Protein Binding

Stability of L-FddCMP-bisSATE in whole blood was determined by spiking whole blood with a 10 μM concentration and incubating aliquots up to 15 minutes at 37° C. Subsequent separation of plasma from cellular components and HPLC analysis revealed 30% decomposition within 5 minutes with no further decomposition up to 15 minutes. *In vitro* and *in vivo* protein binding was determined to be approximately 40% and 45%, respectively.

Kinetics of L-FddCMPbis-SATE and metabolites

Unchanged L-FddCMP-bisSATE was not detected in the plasma at any time following intravenous or oral administration of 3 mg L-FddCMP-bisSATE per kilogram body weight in rhesus monkeys, suggesting rapid and extensive *in vivo* metabolism or degradation of L-FddCMP-bisSATE. The pharmacokinetic parameters of L-FddCMP-bisSATE metabolites are presented in Table 5. The apparent 100% metabolic clearance and zero AUC for L-FddCMP-bisSATE prohibited the determination of total clearance and estimating volume of distribution parameters. L-FddCMP-SH was detected at 0.25 hour in two monkeys, RSa5 and RUa5, at 0.09 and 0.58 μM, respectively. L-FddCMP-SH achieved a C_{Max} of 0.43 μM at 1 hour in monkey

Table 4. Intracellular accumulation of 10μM L-FddCMP-bisSATE metabolites in unstimulated (U) peripheral blood mononuclear cells and peripheral blood mononuclear cells stimulated (S) with 0.1% phytohemagluttanin for 72 hrs.

Compound	Status	Intracellular Concentration (pmol / 10^6 cells)			
		Time (hr)			
		1	4	8	24
L-FddCMP-bisSATE	S	1.1 ± 0.2[a]	1.2 ± 0.2	0.6 ± 0.5	ND[b]
	U	2.5 ± 0.3	1.8 ± 0.3	1.0 ± 0.7	ND
L-FddCMP-monoSATE	S	2.8 ± 0.6	1.9 ± 0.4	1.0 ± 0.7	ND
	U	2.0 ± 0.2	1.7 ± 0.3	1.2 ± 0.9	ND
L-FddCMP-SH	S	19.4 ± 11.2	28.9 ± 7.4	24.0 ± 2.1	ND
	U	20.7 ± 18.3	23.3 ± 5.0	20.6 ± 2.2	3.9 ± 2.2
M I	S	47.5 ± 0.7	47.5 ± 12.0	13.1 ± 0.2	ND
	U	70.2 ± 6.8	85.7 ± 9.0	42.3 ± 6.6	14.8 ± 1.3
L-FddC	S	19.9 ± 3.4	38.9 ± 7.6	30.3 ± 6.9	2.1 ± 1.9
	U	7.5 ± 2.8	17.2 ± 2.6	42.4 ± 5.5	9.3 ± 5.6
L-FddCMP	S	66.1 ± 22.5	89.8 ± 6.6	55.0 ± 5.4	11.8 ± 9.3
	U	46.9 ± 14.4	76.9 ± 19.1	66.2 ± 12.7	73.4 ± 16.2
L-FddCDP-choline	S	1.0 ± 0.4	2.1 ± 0.2	4.8 ± 0.3	0.8 ± 1.2
	U	0.7 ± 0.5	2.7 ± 0.2	3.9 ± 2.1	23.9 ± 0.7
L-FddCDP	S	17.3 ± 3.4	93.1 ± 5.3	121.8 ± 7.8	5.7 ± 1.6
	U	19.7 ± 4.9	132.3 ± 18.9	209.1 ± 34.9	486.1 ± 28.1
L-FddCTP	S	22.2 ± 4.9	177.8 ± 20.5	242.1 ± 13.2	12.8 ± 10.8
	U	10.1 ± 2.8	129.6 ± 19.4	179.3 ± 49.1	410.1 ± 46.2

a: Data are the mean ± S.D. of cells obtained from one donor assessed in triplicate
b: ND = not detected.

RNa5 and declined below our limit of detection (LOD = 0.01 μM) by 2 hours. L-FddCMP-monoSATE was the predominant metabolite in all three monkeys with a variable C_{Max} of 2.06 ± 1.00 μM and an AUC ranging from 1.90 - 6.52 μmol*h/L. The average plasma elimination $T_{1/2}$ of L-FddCMP-monoSATE was 0.66 h. Levels declined below detection at 4 hours in monkey RNa5 (excluded from $T_{1/2}$ data-set) and 6 hour in the other two monkeys. L-FddC achieved a C_{Max} of 0.39 ± 0.15 μM within 0.5 hour, and a detectable level of 0.02 and 0.03 persisted out to 24 hours in monkeys RSa5 and RUa5, respectively. L-FddC exhibited similar

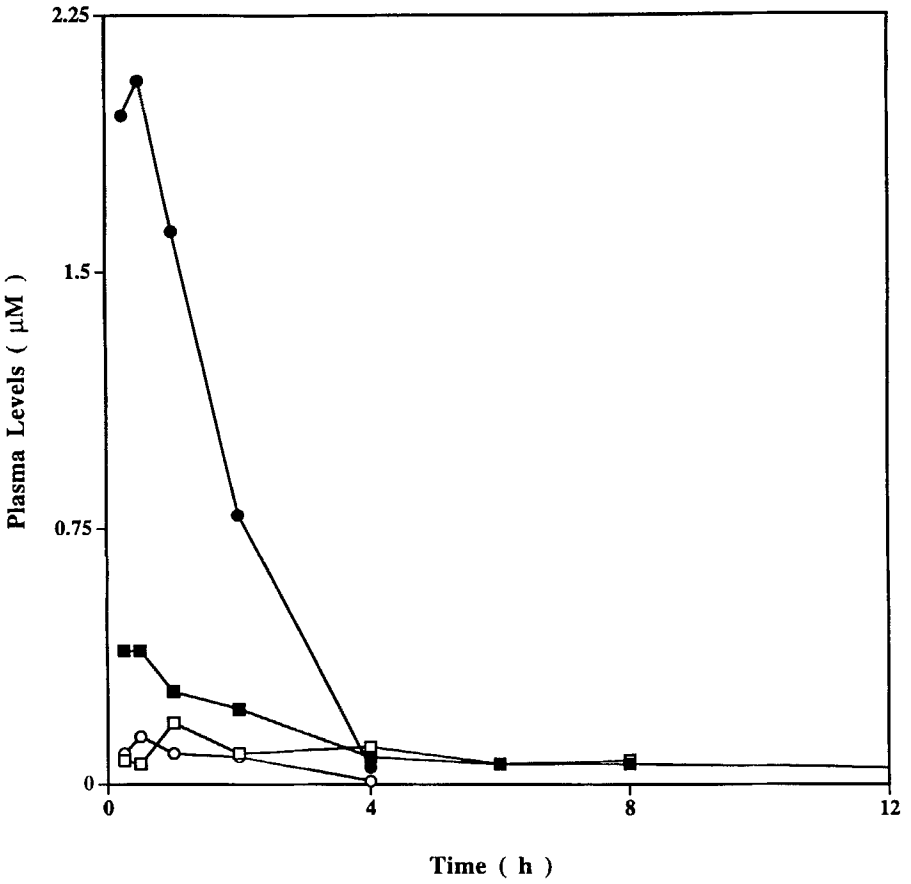

Figure 2. Mean plasma concentration-versus-time L-FddCMP-monoSATE (●, O) and L-FddC (■, [ ]) levels after intravenous (■, ●) and oral (□, O) administration of 3 mg of L-FddCMP-bisSATE per kg body weight.

distribution phase plasma elimination $T_{1/2alpha}$ = 0.9 ± 0.5 h in all three monkeys. Additionally, L-FddC exhibited an extended average terminal-phase elimination $T_{1/2beta}$ = 41.9 h in two monkeys. A peak eluted at 4 minutes which consist of either protein bound MI or protein bound L-FddCMP-SH. The AUC of this entity indicated approximately 45% protein binding. This was independent of dose administration route. Protein binding positively correlated with metabolic elimination of L-FddCMP-monoSATE. The oral bioavailability of L-FddC derived from orally administered L-FddCMP-bisSATE was 13%. Plasma concentration versus time data for L-F-ddCMP-monoSATE and L-FddC plasma levels following administration of 3 mg/kg body weight L-FddCMP-bisSATE is presented in figure 2.

Urinary excretion of L-FddCMP-bisSATE and catabolites

In accordance with plasma data, the unchanged parent drug was not detected in the urine after intravenous and oral administration of L-FddCMP-bisSATE. Rapid elimination of

Table 5. Pharmacokinetic parameters and urinary recovery after intravenous administration of 3 mg of L-FddCMP-bisSATE per kg of body weight in rhesus monkeys.

Monkey	Compound	AUC (h*μmol / L)	CL_R (L / h / kg)	Amnt Excr. (%)	C_{Max} (μM)	T_{Max} (h)	Fd (%)
RNa5	L-FddCMP-monoSATE	2.90	0.24	26.1	1.41	1	NA[a]
	L-FddCMP-SH[c]	NA[b]	NA	30.1	NA	NA	NA
	M I[c]	NA[b]	NA	7.8	NA	NA	NA
	L-FddC	1.14	0.32	28.0	0.31	0.25	12[e]
RSa5	L-FddCMP-monoSATE	1.90	0.15	14.4	2.04	0.5	20[c]
	L-FddCMP-SH[c]	NA[b]	NA	22.3	NA	NA	NA
	M I[c]	NA[b]	NA	19.3	NA	NA	NA
	L-FddC	1.44	0.34	41.8	0.37	0.5	14[e]
RUa5	L-FddCMP-monoSATE	6.52	0.05	14.9	4.61	0.25	NA
	L-FddCMP-SH[c]	NA[b]	NA	30.5	NA	NA	NA
	M I[c]	NA[b]	NA	7.3	NA	NA	NA
	L-FddC	1.84	0.22	46.4	0.60	0.25	14[e]
Mean (± SD)	L-FddCMP-monoSATE	3.77 (1.98)	0.15 (0.08)	18.5 (5.4)	2.69 (1.38)	0.58 (0.31)	20[c]
	L-FddCMP-SH[c]	NA[b]	NA	27.6 (3.8)	NA	NA	NA
	M I[c]	NA[b]	NA	11.5 (5.5)	NA	NA	NA
	L-FddC	1.47 (0.29)	0.29 (0.05)	38.7 (7.8)	0.43 (0.12)	0.33 (0.12)	13[e] (1)

a: NA, Not available.
b: Not detected in the plasma, but excreted in the urine.
c: Calculation of oral bioavailability of L-FddCMP-monoSATE was only possible with monkey RSa5.
d: Oral bioavailability
e: Relative oral bioavailability of L-FddC derived from the prodrug was calculated as described in the pharmacokinetic analysis section of Materials and Methods.

substantial amounts of L-FddCMP-monoSATE, L-FddCMP-SH, and MI were excreted in the urine within 24 h after intravenous and oral dose administration. These catabolites accounted for 18.5 ± 5.4%, 27.6 ± 3.8%, and 11.5 ± 5.5%, respectively, of the excreted radioactivity after intravenous L-F-ddCMP-bisSATE administration. L-FddC accounted for 38.7 ● 7.8% of the excreted radioactivity. Prolonged excretion of radioactivity was observed in the monkeys out to 7 to 11 days. This radioactivity was confirmed to be L-FddC by HPLC analysis (retention time) and MS/MS and parent ion MS. After oral administration of L-FddCMP-bisSATE, approximately equal percentages of L-FddCMP-monoSATE and MI were detected in the urine. However, a greater percentage of L-FddC and a smaller percentage of L-FddCMP-SH was excreted in the urine after oral administration of L-FddCMP-bisSATE when compared with the

intravenous route. This may reflect extensive gastrointestinal catabolism of the sulfhydryl derivative. Renal clearance (CL_R) of L-FddCMP-monoSATE and L-FddC was 0.15 ± 0.08 and 0.29 ± 0.05 L/hr/kg. These results are presented in Table 5.

DISCUSSION

Intracellular nucleoside-triphosphate accumulation is a function of sequential phosphorylation of the parent nucleoside determined by its respective substrate affinity for anabolic kinases and the inherent stability of the 5'-triphosphate as dictated by its intrinsic stability to catabolic phosphatases and phosphodiesterases. Additionally, the antiviral activity of the 5'-triphosphate is a function of its intracellular level and its affinity for the viral polymerase. Previous reports confirm that L-FddC is a potent and selective inhibitor of HIV and HBV replication, *in vitro*.[18,19] It exhibited an anti-HIV activity with an EC_{50} = 0.02 μM in PBM and CEM cells with selectivity exceeding 3100 in each respective cell line. Interestingly, the anti-HIV activity was approximately 40-fold lower (EC_{50} = 0.8 μM) in MOLT-4 cells. The substantial difference in anti-HIV activity of L-FddC in CEM and MOLT-4 cells may reflect variations in viral replication dynamics in these different acute lymphoblastic leukemia cell lines. Under identical conditions, the anti-HIV activity of L-FddCMP-bisSATE was comparable with an EC_{50} = 0.05 μM in CEM cells and 0.9 μM in MOLT-4 cells. However, an approximate 2-fold toxicity increase was observed with the L-FddCMP-bisSATE prodrug when compared with the parent nucleoside. This data, in conjunction with L-FddCTP's observed K_i of 1.6 ± 0.1 μM for the inhibition of dCTP incorporation into an appropriate template-primer by HIV-RT may indicate that intracellular L-FddCTP level present in L-FddC exposed cells is sufficient to suppress viral replication and that HIV-RT may be saturated at this intracellular concentration or that the SATE moiety introduces itself a minor degree of *in vitro* toxicity.[27] Similar to *in vitro* lymphocyte studies, L-FddCMP-bisSATE (IC_{50} = 20 μM) was more toxic in Hep-G2 cells when compared with L-FddC (IC_{50} = 438 ± 57 μM).[19] The anti-HBV activity assessed by reduction in intracellular replicative intermediate did not change dramatically between the nucleoside and its prodrug.

L-FddCMP-bisSATE cellular pharmacology investigations in a variety of cell types demonstrates rapid passive diffusion and intracellular liberation of L-FddCMP with subsequent generation of high intracellular L-FddCTP levels. Direct intracellular delivery of L-FddCMP, *in vitro*, increased over 4-fold the intracellular accumulation of L-FddCTP when compared with equivalent molar L-FddC exposure in Hep-G2 cells.[20] In addition, L-FddCTP derived from the bisSATE prodrug obtained a maximum concentration faster than when cells were exposed to the parent nucleoside. Maximum triphosphate levels reflected significant interindividual variations in nucleoside analog phosphorylation in primary cultured human hepatocytes, *in vitro*, as previously documented in lamivudine treated primary cultured human hepatocytes.[28] Substantial L-FddCTP levels were also achieved in dividing and quiescent peripheral blood mononuclear cells. Cell viability appeared to decline at 24 hours in stimulated PBM cells exposed to 10 μM L-FddCMP-bisSATE while metabolite concentration levels did not decline in unstimulated cells and no toxicity was observed. This toxicity may reflect incorporation of L-FddCTP into DNA of dividing cells, but Faraj et. al. reported that L-FddCTP was not a substrate

for DNA polymerase alpha or beta at concentrations up to 100 μM *in vitro*.[27] Intracellular L-FddCDP-choline was detected in all cell types examined. The endogenous dCDP-choline is involved in phosphatidylcholine biosynthesis.[29] Similar choline derivatives have been previously identified with a variety of cytidine nucleoside analogs used in antiviral and cancer chemotherapy.[30-32] This activated L-FddC liponucleotide derivative was identified in earlier studies by ^{3}H and ^{14}C double labeling experiments, but the substrate, either L-FddCMP or L-FddCTP, consumed to generate L-FddCDP-choline could not be identified. Intracellular levels of the 5'-phosphorylated derivatives and L-FddCDP-choline in conjunction with their respective peak concentration times suggest that the diphosphocholine derivative is formed from the 5'-triphosphate and not the 5'-monophosphate.

In addition to the L-FddC-5'-phosphorylated derivatives, the corresponding mono-SATE and sulfhydryl derivatives and one unidentified metabolite, M I, of L-FddCMP-bisSATE were detected in both the intracellular and extracellular compartments in all cell types. These metabolites were identified based on decomposition studies of L-ddAMP-bisSATE.[33] Intracellular prodrug and catabolite levels indicate extremely rapid passive diffusion of L-FddCMP-bisSATE into the cytosol with subsequent rapid decomposition to L-FddCMP-monoSATE. Further metabolism to the sulfhydryl derivative occurs slower than the initial decomposition, but this does not compromise the ultimate liberation of L-FddCMP. Extracellular catabolite levels indicate efflux of a portion of L-FddCMP-monoSATE, L-FddCMP-SH, and MI as they are generated. However, this extracellular directed equilibrium still permits the accumulation of substantial L-FddCMP which is sequentially phosphorylated by cytidylate kinase and nucleoside diphosphokinase.

Previously, our group evaluated the pharmacokinetics of L-FddC in rhesus monkeys.[21] Based on plasma and urine data after intravenous and oral administration of a 5 mg per kilogram of body weight dose of L-FddC, L-FddC exhibited a biphasic elimination from the plasma with a terminal elimination $T_{1/2beta} = 1.8 \pm 0.2$ hours and a total clearance of 0.7 ± 0.1 L/h/kg. There was no detectable metabolism of L-FddC *in vivo*, and the volume of distribution indicated substantial distribution of L-FddC outside the systemic vasculature. L-FddC exhibited an oral bioavailabity in these monkeys between 56 and 66%, and only 47 ± 16 % of the intravenously administered dose was recovered in the urine.[21] The inclusion of biolabile groups has improved the oral bioavailability of some nucleoside analogs. This strategy has been successfully implemented with the antiherpetic drug penciclovir and the anti-HIV drug PMEA.[23,34] Their orally administered prodrugs, famciclovir and adefovir dipivoxil employ different chemical modifications that mask the anionic nature of the parent acyclic nucleoside analogs. Famciclovir has neutralized negative charges with acetyl groups that are cleaved by endogenous esterases with further hepatic extraction and oxidation of the base to yield the corresponding guanine base and orally bioavailable penciclovir. *In vitro* anti-herpes simplex virus (HSV) studies documented an approximate equivalent antiviral effect.[34] However, oral bioavaibility of penciclovir was approximately 5% in mice after oral administration of penciclovir.[35] Administration of famciclovir increased the oral bioavailability of penciclovir to 77% in humans.[34] The increased oral bioavailability permits reduction in the orally administered penciclovir dose with a decreased toxicity and an equivalent therapeutic benefit. This premise

was also the initiative for the development of adefovir dipivoxil (bis-POM-PMEA) and apropovir disprometil (bis-POC-PMPA). Chemical modification incorporating biolabile ester-linked POM and POC protecting groups on Adefovir (PMEA) and Apropovir (PMPA), respectively, has been implemented to increase the oral bioavailability of these acyclic nucleoside analogs.

Successful implementation of biolabile protecting groups to increase the oral bioavailability of nucleotides prompted our group to assess the effect of inclusion of biolabile (t-butyl)SATE protecting groups on the absolute oral bioavailability of the parent nucleoside, L-FddC, and to determine the *in vivo* metabolism of L-FddCMP-bisSATE. *In vivo* metabolism was qualitatively similar to *in vitro* studies. Plasma and urine data indicated 100% metabolic clearance of L-FddCMP-bisSATE from plasma within 15 minutes. Whether this is due to significant concomitant metabolism during gastrointestinal absorption in conjunction with high hepatic extraction and rapid renal clearance within the first 15 minutes is yet unclear. However, substantial amounts of L-FddCMP-SH and M I were excreted in the urine, but were not detected in the plasma. Alternatively, protein binding of L-FddCMP-monoSATE, L-FddCMP-SH, or M I which was approximately 45% may interfered with the absolute quantification of these metabolites. Proteinuria was excluded by urine Bradford analysis. Interestingly, maximum protein binding occurred within 5 hours after dose administration and correlated with clearance of L-FddCMPmonoSATE from plasma. Assuming that L-FddCMP-SH and M I are derived from L-FddCMP-monoSATE, it may very well be these derivatives that are bound to proteins with a molecular weight greater than 30,000 daltons.[36] Radioactivity was detected in the urine until 7 to 11 days after both intravenous and oral L-FddCMP-bisSATE dosage in monkeys. Radioactivity was isolated by HPLC and subjected to mass spectrometry. MS/MS and parent-ion MS confirmed the identity of the radioactivity as L-FddC. Prolonged excretion of L-FddC may be explained by either a decrease in clearance over time, or a more feasible explanation may be the extended intracellular half-life of L-FddCTP. The oral bioavailability of L-FddC when administered as its prodrug, L-FddCMP-bisSATE, is approximately 65% which is comparable to the oral bioavailability of administered L-FddC.[21] However, the relative amount of L-FddC delivered when L-FddCMP-bisSATE is administered and accounting for the difference in the molecular weight of L-FddCMP-bisSATE and L-FddC is approximately 5-fold less than when the parent nucleoside is administered. This can be attributed to the renal clearance of L-FddCMP-monoSATE, L-FddCMP-SH, and M I. These metabolites accounted for 18.5 ± 5.4%, 27.6 ± 3.8%, and 11.5 ± 5.5%, respectively, of the excreted radioactivity. Renal clearance for L-FddCMP-monoSATE was 0.15 ± 0.08 L/h/kg which is approximately equal to creatinine clearance indicating elimination primarily by glomerulus filtration with minor or no reabsorption.[37] Renal clearance of L-FddC (CL_R = 0.29 ± 0.05 L/h/kg) exceeded creatinine clearance confirming that in addition to glomerulus filtration, L-FddC undergoes active renal tubular secretion.[37]

In summary, L-FddCMP-bisSATE rapidly diffuses into Hep-G2, stimulated and unstimulated human hepatocytes, and PHA-stimulated PBM cells in culture with subsequent catabolism to L-FddCMP-monoSATE, L-FddCMP-SH, MI, L-FddCMP, and the parent nucleoside L-FddC. The initial esterase mediated catabolism of L-FddCMP-bisSATE to L-

FddCMP-monoSATE occurs faster than the carboxyesterase mediated production of L-FddCMP-SH. Intracellular L-FddCMP, L-FddCDP, and L-FddCTP suggest that deoxycytidine kinase is the rate limiting enzyme in the intracellular activation of L-FddC to its corresponding 5'-triphosphate derivative. Furthermore, cells exposed to L-FddCMP-bisSATE when compared to cells exposed to L-FddC, accumulate approximately 3-fold more L-FddCTP. However, the anti-HIV and anti-HBV activities of L-FddCMP-bisSATE and L-FddC and their corresponding selectivity does not proportionately increase. *In vitro* metabolism of L-FddCMP-bisSATE was predictive of *in vivo* metabolism. Furthermore, rapid metabolism of L-FddCMP-bisSATE to its corresponding monoSATE and sulfhydryl derivative in conjunction with protein binding led to significant urinary clearance of L-FddC intermediates and approximately 80% less systemic exposure when compared to administration of the parent nucleoside. Further studies are necessary to extend these observations regarding the *in vivo* potential of the SATE strategy.

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