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Preclinical and clinical development of the anti-HIV, anti-HBV oxathiolane nucleoside analog emtricitabine

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Introduction

Acquired immunodeficiency syndrome (AIDS) was first described in the United States in 1981, with the unexplained appearance of Kaposi's sarcoma, *Pneumocystis carinii* pneumonia and other opportunistic infections in previously healthy homosexual males [1]. Affected individuals became susceptible to opportunistic infections and specific immune deficiency resulting from the depletion of $CD4^+$ lymphocytes. Intensive investigation revealed the etiologic agent of AIDS to be a lymphotropic retrovirus, the human immunodeficiency virus (HIV) [2, 3]. Today, three classes of drugs are available to treat patients infected with HIV: the nucleoside reverse transcriptase inhibitors (NRTI), the nonnucleoside reverse transcriptase inhibitors (NNRTI), and the protease inhibitors (PI). The currently accepted standard of care for HIV infection involves the use of three drug combination regimens [4]. The use of combination therapy has profoundly reduced the morbidity and mortality associated with HIV infection. However, the approved anti-HIV drugs and the combination(s) of these drugs have significant limitations including toxicity, the selection of drug-resistant variants, pharmacokinetic interactions with other agents and poor adherence due to complex dosing regimens. These limitations have necessitated the continued search for anti-HIV agents with an improved clinical profile.

Hepatitis B virus (HBV) also constitutes a major worldwide health threat. In addition to the morbidity associated with acute clinical infection, chronic liver disease, cirrhosis, hepatitis delta virus infection and primary hepatocellular carcinoma are recognized sequelae [5]. The reservoir for HBV includes an estimated worldwide population of 300 million carriers. Approximately 25% of chronic carriers will die from primary hepatocellular carcinoma or cirrhosis of the liver. At present there are only two approved therapies for the treatment of HBV infection, α-interferon and lamivudine. The limited efficacy of α-interferon and the emergence of lamivudine-resistant HBV variants have made it clear that additional therapies are needed for the treatment of acute and chronic

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HBV infection. These new therapies will include combination regimens analogous to those used to treat HIV infections.

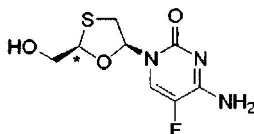

Figure 1. Structure of emtricitabine (524W91, FTC) [6]. The oxathiolane ring, which is an analog of a 2',3'-dideoxyribose ring, has the L configuration at what corresponds to the 4' position (*). This is in contrast to naturally occurring nucleosides, which have a D configuration at this position.

The 2',3'-dideoxynucleoside analog (2R,5S)-5-fluoro-1-[2-(hydroxymethyl)-1,3-oxathiolan-5-yl]cytosine (Figure 1) is a potent and selective inhibitor of HIV and HBV replication *in vitro* and *in vivo*. Clinical studies have demonstrated the drug to be very effective in suppressing both HIV and HBV in infected patients. In this report we will present an overview of the preclinical and clinical studies conducted to date on emtricitabine and when possible critique differences between emtricitabine and other nucleoside analogs currently approved for the treatment of HIV and HBV disease. Particular attention will be paid to comparing the antiviral activity of emtricitabine to that of lamivudine and differentiating the activities of the two compounds based on the introduction of fluorine into the C-5 position of emtricitabine.

In vitro antiviral activity

The antiviral activity of emtricitabine has been shown to be specific for HIV-1, HIV-2, and HBV. No activity has been observed at concentrations up to 100 μM against HSV-1, HSV-2, HCMV, VZV, coronavirus, yellow fever virus, respiratory syncytial virus, rotavirus, influenza virus, or rhinovirus [7].

Anti-HIV activity

The ability of emtricitabine to inhibit replication of HIV-1 and HIV-2 in cell culture has been studied extensively using various human T-lymphoid cell lines (MT-2, MT-4, CEM and HT4-6C), and PMBCs infected with laboratory-adapted strains of HIV-1 (III_B, LAI or LAV) and HIV-2 (ZY, ROD2). The results are summarized in Table 1. A comparison of the *in vitro* potency of emtricitabine to that of the other nucleoside analogues currently used to treat HIV is difficult owing to the wide variety of cell types, virus strains and assay conditions used by the numerous investigators who have determined EC_{50} values for these compounds. However, if EC_{50} data derived from PBMCs infected with the LAI, LAV or III_B strain of HIV-1 are used as the basis of comparison, emtricitabine and the nucleoside analogues currently approved for

human use can be divided into three potency groups. The most potent group includes emtricitabine and AZT, with EC_{50} value ranges of 0.001 to 0.01 μM and 0.001 to 0.058 μM, respectively. The second potency group includes d4T, 3TC and ddC, with EC_{50} value ranges of 0.04 to 0.09 μM, 0.04 to 0.53 μM, and 0.01 to 0.23 μM, respectively. The least potent group includes ddI and abacavir, with EC_{50} value ranges of 0.46 to 19 μM, and 3.7 μM, respectively.

Table 1. Inhibitory effect of emtricitabine on the replication of laboratory strains of HIV-1 and HIV-2

Virus (strain)	Cell Type	EC_{50} (μM)
HIV-1 (IIIB)	CEM	0.1[a,b]
	MT-4	0.5[a,b]
	PBMC	0.01[b]
HIV-1 (LAV)	CEM	0.009[a]
	HT4-6C	0.02[a]
	PBMC	0.009[b], 0.001[d]
HIV-1 (LAI)	CEM	0.04[c]
	MT-2 PBMC	0.62[c], 0.001[e]
	PBMC	0.03[c]
HIV-2 (2ZY)	MT4	1.5[a]
	CEM	0.1[a]
HIV-2 (ROD2)	PBMC	0.007[b]

a. [8]
b. [7]
c. Personal Communication, K. Borroto-Esoda, Triangle Pharmaceuticals
d. [9]
e. [10]

Several investigators have compared the anti-HIV activity of emtricitabine with that of lamivudine in the same assay, thereby eliminating any ambiguities introduced by an interassay comparison. The results are summarized in Table 2. Although different laboratory strains of virus, different cell types and different assay methods were used by the investigators in these studies, emtricitabine consistently showed greater activity than lamivudine with the activity advantage ranging from three- to 11-fold.

Table 2. Comparison of the anti-HIV activity of emtricitabine (FTC) with lamivudine (3TC) using various laboratory strains of HIV-1

		EC_{50} (μM)		
HIV subtype	Cell Line	FTC	3TC	Sensitivity Ratio[d]
LAI[a]	PBMC	0.018	0.19	11
IIIB[b]	PBMC	0.01	0.07	7
IIIB[b]	MT4	0.5	3.2	6
LAI[a]	MT2	0.3	1.6	5
HXB2[c]	MT4	0.09	0.24	3
LAI[a]	$CD4^+$ HeLa	0.06	0.18	4

a. Personal communication, D. Wakefield, Triangle Pharmaceuticals
b. [8]
c. [11]
d. Defined as the quotient of EC_{50} (3TC)/EC_{50} (FTC)

The sensitivity of HIV-1 clinical isolates to inhibition by anti-HIV drugs is assessed *in vitro* to gain insight into the variation in activity that might be encountered in a clinical setting. The results of one such study conducted with emtricitabine in human PBMCs are presented in Table 3. Schinazi et al. tested two low passage clinical isolates; J6 and 2:DR2, in phytohemagglutinin-stimulated PBMCs isolated from uninfected donors [8]. The EC_{50} values are similar to those calculated using laboratory strains of virus in PBMCs. The sensitivities to emtricitabine reported for two additional wild type clinical isolates, WT-pre-AZT (obtained from D. Richman, Veterans Affairs Medical Center, San Diego, CA) and WT-MKC09-day 29 (obtained from a Phase I Emivirine trial, Triangle Pharmaceuticals, Inc.), were similar to those determined by Schinazi et al. against J6 and 2:DR2. In the single experiment in which a direct comparison was made between the activity of emtricitabine and lamivudine, emtricitabine demonstrated the same fivefold potency advantage seen using laboratory strains of virus.

The potency of emtricitabine has also been determined using a coculture assay [12] and compared directly to the potencies of lamivudine, zalcitabine, didanosine, zidovudine, and the non-nucleoside RT inhibitor TIBO. In this study, PBMCs from HIV-infected patients were cocultured with PBMCs isolated from uninfected donors. The naturally infected PBMCs served as a source of a diversified population of virus not selected for by *in vitro* propagation. At the end of the coculture period, the degree of viral replication was measured by HIV-1 p24 ELISA. Results from this study expressed as mean EC_{50}, EC_{90}, and EC_{99} values are given in Table 4. A potency ranking (based on EC_{90} values) showed emtricitabine to be the most potent compound, followed by zalcitabine, lamivudine, zidovudine, TIBO, and didanosine. The low potency ranking for zidovudine compared to that observed in laboratory strains may be the result of inclusion of PMBCs from AZT-experienced patents in the coculture.

Table 3. Inhibition of HIV-1 clinical isolates by emtricitabine

Virus	EC_{50} (μM)	
	emtricitabine	lamivudine
J6[a]	0.002	0.01
2:DR2[a]	0.002	ND
WT-pre-AZT[b]	0.008	ND
WT-MKC09-day 29[b]	0.02	ND

a. [8]

b. Personal communication, K. Borroto-Esoda, Triangle Pharmaceuticals

ND. Not Determined

Table 4. Comparative potency of RT inhibitors in HIV-1-infected PBMCs using a cocultured method [a]

Inhibitor	EC_{50} (μM)	EC_{90} (μM)	EC_{99} (μM)
Emtricitabine	0.0085	0.055	0.43
3TC	0.11	0.3	0.85
ddC	0.011	0.074	0.6
ddI	0.76	6.4	65.8
AZT	0.055	0.53	6.4
TIBO R82913	0.17	0.67	2.95

a. [12]

To determine if the antiviral activity of emtricitabine varied in different subtypes of HIV-1, activity was evaluated against HIV-1 group M and group O in MAGI-CCR5 cells and PBMCs. Table 5 shows the results. EC_{50} values were also obtained for lamivudine, zidovudine, and didanosine. Emtricitabine was more active than lamivudine and didanosine, and had activity comparable to that of zidovudine.

Table 5. EC_{50} values of NRTIs against HIV-1 group M and O in PBMCs and MAGI cells [a]

Isolate	Subtype	Host Cell	AZT	3TC	ddI	FTC
Group M						
UG/92/024	D	PBMCs	0.003	0.026	0.21	0.007
BR/92/025	C	PBMCs	0.035	0.027	0.49	0.017
RW/92/008	A	PBMCs	0.008	0.054	0.26	0.012
Tha/92/019	E	PBMCs	0.039	0.069	0.5)	0.028
Br/930/20	F	PBMCs	0.003	0.022	0.34	0.009
RU570	G	PBMCs	0.008	0.090	0.34	0.030
Group O						
BCF02 O	O	PBMCs	0.028	2.5	4.75	0.14
Group M						
RW/92/008	A	MAGI	0.085	0.20	3.0	0.055
BR/92/025	C	MAGI	0.033	0.17	0.95	0.032
UG/92/024	D	MAGI	0.035	0.11	1.70	0.030
Tha/92/019	E	MAGI	0.080	0.15	1.50	0.065
Br/930/20	F	MAGI	0.045	0.15	1.50	0.050
RU570	G	MAGI	0.150	0.18	2.50	0.075
Group O						
BCF03 O	O	MAGI	0.09	0.20	2.20	0.065

a. Personal communication, D. Wakefield and S. Fleming, Triangle Pharmaceuticals

Anti-HBV activity

The *in vitro* anti-HBV activity of emtricitabine has been studied extensively using the stably HBV-transfected cell line HepG2 2.2.15. In this system, emtricitabine decreases levels of extra- and intracellular HBV DNA in a dose-dependent manner. EC_{50} values determined by various investigators using extracellular HBV DNA levels range from 0.01 ± 0.005 to 0.04 ± 0.001. The emtricitabine EC_{50} value based on intracellular DNA reported by Schinazi et al. [13] is somewhat higher, 0.16 ± 0.01 μM, than the EC_{50} values based on extracellular DNA (Table 6) [14]. For comparison EC_{50} values of other nucleoside analogues currently under development as anti-HBV agents are presented along with those of emtricitabine and lamivudine in Table 6. In contrast to HIV, the EC_{50} values for emtricitabine and lamivudine are similar against HBV.

Table 6. Anti-HBV activity in HepG2 2.2.15 of compounds approved and under development for HBV infection

Compound	EC_{50} values		
Emtricitabine	0.01 ± 0.005 μM[a]	0.075 ± 0.009 μM[d]	0.04 ± 0.006 μM[b, f]
Lamivudine	0.008 ± 0.003 μM[a]	0.05 ± 0.01 μM	
Adefovir	0.03 ± 0.01 μg/ml[c]		
Clevudine	0.1 ± 0.06 μg/ml[c]	0.38 ± 0.25 μM[d]	0.296 ± 0.036 μM[e]
Tenofovir	0.04 ± 0.02 μg/ml[c]		
Lobucavir	0.1 ± 0.1 μg/ml[c]		
Penciclovir	3.5 ± 0.2 μg/ml[c]		
Amdoxovir	13 ± 2.1 μg/ml[c]		
Entecavir	0.004 μM[g]		
Epavudine (L-dT)	0.19 ± 0.09 μM		
Epcitabine (L-dC)	0.24 ± 0.08 μM[f]		
β-L-Fd4C	<0.1 μM[f]		

a. [14]
b. [13]
c. [15, 16]
d. Personal communication W. Nicholson, Triangle Pharmaceuticals
e. Personal communication B. Korba, Division of Molecular Virology and Immunology, Georgetown University
f. [17]
g. [18]

Condreay et al. examined the effect of emtricitabine on HBV replication in primary human hepatocytes [19]. Although EC_{50} values were not calculated, emtricitabine at 2 μM completely inhibited the production of intracellular HBV DNA, even when added 24 hours after infection. The EC_{50} value calculated from inhibition of extracellular virus production is <0.02 μM, a value that is comparable to that determined in HepG2 2.2.15 cells (Table 6).

Mechanism of action

Cellular uptake

Transport studies were conducted in confluent cultures of HepG2 2.2.15 cells to determine the route(s) of cellular uptake of emtricitabine [20]. Assays were performed at room temperature (20°C) using a modified rapid, cold buffer stop method. The influx of emtricitabine into the cells did not depend on the concentration of emtricitabine or the presence of a Na^+ gradient, and was only partially inhibited by competing nucleosides

and nucleoside transport inhibitors (Table 7). The negligible impact of the protein modification agents N-ethylmaleimide, 4,4'-diisothiocyanato-2, 2'-stilbenedisulfonic acid and 4-aceamido-4'-isothiocynatostilbene-2, 2'-disulfonic acid on emtricitabine uptake suggests that a component of emtricitabine entry into HepG2 2.2.15 cells was not transporter mediated, but possibly results from non-facilitated diffusion.

Table 7. Inhibition of emtricitabine influx into cells by nucleoside transport inhibitors, competing nucleosides, and adenine[a, b]

Agent	Concentration (μM)	Inhibition (%)	Experiments (#)
NBMPR	10	45	6
Dipyridamole	10	48	6
Dilazep	10	35	6
Cytidine	500	37	3
Uridine	500	69	2
Inosine	500	60	2
Adenine	500	14	3

a. [20]
b. Experiments were performed in HepG2 2.2.15 cells in the presence of 10 μM emtricitabine

Anabolism

Emtricitabine is efficiently phosphorylated in HepG2 2.2.15 cells to the corresponding 5'-mono, 5'-di and 5'-triphosphates. A time course showed that the nucleotides of emtricitabine were formed rapidly and reached a steady-state intracellular concentration by three to six hours [20]. The concentration of emtricitabine 5'-diphosphate was somewhat higher than the concentration of the 5'-mono and 5'-triphosphate derivatives [7, 14, 20, 21], as shown in Figure 2. When the intracellular concentrations of the phosphorylated forms were measured as a function of the extracellular concentration (concentrations ranged from 0.01 to 10 μM), the concentration of the 5'-phosphorylated derivatives of emtricitabine increased in a dose-dependent manner, indicating that the anabolic pathway was not saturated at the concentrations tested (Figure 2). An intracellular half-life of approximately 2.4 hours was determined for emtricitabine 5'-triphosphate in HepG2 cells [20]. This half-life is extremely short compared to the estimated 30 hours half-life seen in PBMCs taken from healthy human volunteers dosed orally with 200 mg emtricitabine QD [22].

In studies to determine which enzymes were responsible for phosphorylating emtricitabine to the 5'-triphosphate, 2'-deoxycytidine kinase was identified as the enzyme that catalyzes the phosphorylation of emtricitabine to the corresponding 5'-monophosphate [14, 21, 23]. Using calf thymus 2'-deoxycytidine kinase, the relative

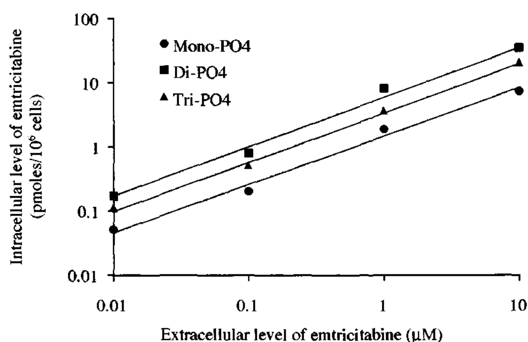

Figure 2. A $\log_{10}$ plot of the intracellular concentrations of emtricitabine 5'-phosphates as a function of extracellular emtricitabine ((●)-5'-monophosphate, (■)-5'-diphosphate, (▲) –5'-triphosphate) [20]. In this experiment HepG2 2.2.15 cells were incubated with emtricitabine for 24 hours.

rate of phosphorylation was 3.4% of that measured for 2'-deoxyguanosine and 40% of that observed for 2'-deoxycytidine. Shewach et al. demonstrated emtricitabine to be a good substrate for human deoxycytidine kinase [23]. An apparent K_m value of 11.8 μM and an apparent relative V_{max} of 9.3 nmol·mL^{-1}·hr^{-1} was determined when UTP was the phosphate donor. When ATP was the phosphate donor, an apparent K_m value of 7.4 μM and an apparent relative V_{max} of 3.8 nmol·mL^{-1}·hr^{-1} was obtained. Phosphorylation of the 5'-monophosphate was catalyzed by 2'-deoxycytidine monophosphate kinase [14, 21]. The efficiency with which the enzyme, purified from calf thymus, phosphorylates the 5'-monophosphate of emtricitabine was approximately 32% of that observed when using the natural substrate, 2'-deoxycytidine 5'-monophosphate. Emtricitabine 5'-monophosphate can also be phosphorylated by nucleoside monophosphate kinase purified from beef liver. However, the phosphorylation catalyzed by this enzyme is relatively inefficient. The formation of the 5'-triphosphate of emtricitabine from the 5'-diphosphate has been proposed to be catalyzed by nucleoside diphosphate kinase (NDP) [14], a cytosolic enzyme with a broad specificity for nucleoside 5'-diphosphates. However, Cheng et al. have recently suggested that the L-nucleotide 5'-diphosphates cannot be utilized as a substrate by NDP kinases but are selectively phosphorylated by 3-phosphoglyceride kinase. [24]

Inhibition of HIV-1 reverse transcriptase by the 5'-triphosphate of emtricitabine

Human immunodeficiency virus encodes a reverse transcriptase (HIV-RT) that synthesizes a double-stranded DNA copy of genomic RNA. Emtricitabine 5'-triphosphate serves as an alternative substrate inhibitor of HIV-RT and is incorporated into a growing chain of viral DNA. Incorporation results in the termination of nascent chain DNA synthesis due to the lack of a hydroxyl group in the 3'-position of the sugar moiety of emtricitabine, which in turn results in inhibition of viral replication.

Steady state kinetic experiments comparing emtricitabine 5'-triphosphate with the natural substrate dCTP showed the two substrates to have similar K_m values for HIV-RT (13 nM for emtricitabine 5'-triphosphate and 70 nM for dCTP). The K_i values for emtricitabine 5'-triphosphate inhibition of HIV-RT-catalyzed RNA-dependent DNA synthesis and DNA-dependent DNA synthesis were calculated to be 0.6 μM and 0.43 μM, respectively. In comparison, the K_i values for lamivudine 5'-triphosphate inhibition of HIV-RT-catalyzed RNA-dependent and DNA-dependent DNA synthesis were 0.97 and 0.7 μM, respectively.

Although a steady state kinetic analysis represents a useful beginning, it is insufficient to establish a detailed kinetic and mechanistic picture of the enzyme-catalyzed reaction [25]. A pre-steady-state kinetic analysis can provide a detailed picture of the events that occur at the enzyme active site. This includes binding of the nucleotide substrate to the enzyme-DNA complex to form an initial ternary complex (K_D), the maximum rate of incorporation of the single nucleotide 5'-monophosphate (k_{pol}), and the overall efficiency of incorporation, which is defined as the quotient k_{pol}/K_d. Using rapid quench techniques to carry out a pre-steady-state analysis, Feng et al. compared the pre-steady-state kinetics of single nucleotide incorporation of dCTP, lamivudine 5'-triphosphate and emtricitabine 5'-triphosphate opposite a template guanosine in RNA-dependent DNA synthesis with HIV-1 RT [26]. The results are shown in Table 8. The overall incorporation rate of the oxathiolane nucleoside analogs is significantly slower than that observed for the natural substrate dCTP, as evidenced by the values of k_{pol}.

Table 8. Incorporation of deoxycytidine 5'-triphosphate, emtricitabine 5'-triphosphate and lamivudine 5'-triphosphate into an RNA/DNA template/primer

Compound	Template/Primer	k_{pol} (s^{-1})	K_d (μM)	k_{pol}/K_d (μM·1s^{-1})
2'-Deoxycytidine 5'-triphosphate	R44/D23[a]	9 ± 2	16 ± 5	-
	R45/D23[b]	23 ± 1	30 ± 4	0.76
Emtricitabine 5'-triphosphate	R44/D23[a]	0.240 ± 0.02	1.7 ± 0.3	-
	R45/D23[b]	0.082 ± 0.005	1.4 ± 0.4	0.06
Lamivudine 5'-triphosphate	R45/D23[b]	0.033 ± 0.002	5.0 ± 0.8	0.0067

a. [27]
b. [26]

The k_{pol} value of dCTP ranges from 280 to 700 times greater than the corresponding k_{pol} values for the oxathiolane nucleoside analogues. However, the K_d values reveal that the oxathiolane substrates bind much tighter to the enzyme-DNA complex than does the natural substrate, with the K_d values of the analogues being approximately six- to 30-fold lower than those of the natural substrate. Both the K_d and k_{pol} values reveal emtricitabine 5'-triphosphate to be a better overall substrate for the enzyme than

is lamivudine 5'-triphosphate. The result of the k_{pol} and K_d advantage is increased efficiency for emtricitabine 5'-triphosphate relative to lamivudine 5'-triphosphate. Emtricitabine 5'-triphosphate is incorporated almost an order of magnitude more efficiently than is lamivudine 5'-triphosphate during RNA-dependent DNA synthesis. This efficiency advantage can account in part for the higher activity seen for emtricitabine compared to lamivudine in cell culture (Table 2).

Inhibition of HBV DNA polymerase by the 5'-triphosphate of emtricitabine

The replication cycle of hepadnavirus includes the reverse transcription of an RNA template [28]. This process is carried out by a polymerase that shares significant sequence homology with the RT of retroviruses, including HIV [29]. Since all attempts to date to purify the human HBV DNA polymerase have been unsuccessful, examination of the effect of emtricitabine 5'-triphosphate on HBV DNA polymerase was carried out using an endogenous polymerase assay. In this assay intact virus particles are treated with 1% Nonidet-P40, a nonionic detergent that partially disrupts the virus particles and allows nucleotide substrates to enter the virus particle so that DNA synthesis can occur [14, 30]. HBV particles purified from culture supernatants and treated with detergent have been shown to incorporate the 5'-triphosphate of emtricitabine and to inhibit product formation in a dose-dependent fashion. Competition studies were performed to determine whether the 5'-triphosphate of emtricitabine competes only with dCTP for binding to the enzyme or with other 2'-deoxynucleoside 5'-triphosphate substrates as well. In these experiments, the ability of increasing concentrations of dCTP, dTTP or dGTP to block inhibition of DNA synthesis by emtricitabine 5'-triphosphate was examined. While increased concentrations of dTTP and dGTP had no effect on inhibition by emtricitabine 5'-triphosphate, a tenfold excess of dCTP completely blocked the ability of emtricitabine to inhibit DNA synthesis.

Davis et al., using the endogenous polymerase assay, demonstrated that the HBV DNA polymerase could incorporate [α-^{32}P] emtricitabine 5'-triphosphate into minus strand DNA [30]. In this study [α-^{32}P] emtricitabine 5'-triphosphate was incubated with HBV viral particles (2 x 10^{10}) isolated from HepG2 2.2.15 cell cultures, unlabeled dATP, dGTP, and dCTP. The radiolabeled DNA product was detected by autoradiography. A labeled 3.2 kb DNA was detected indicating that radiolabeled emtricitabine 5'-monophosphate was incorporated into the viral DNA. Endogenous polymerase assays using HBV virus particles isolated from cell cultures treated with emtricitabine 5'-triphosphate showed either reduced or no polymerase activity depending on the concentration of emtricitabine 5'-triphosphate used, even though they had detectable HBV minus strand DNA. Furthermore, the particles produced in emtricitabine 5'-triphosphate-treated cells did not contain any detectable HBV plus strand DNA, which is consistent with the incorporation and chain terminating activity of emtricitabine. Taken together, the results demonstrate that emtricitabine 5'-triphosphate serves as an alternative substrate inhibitor of the HBV DNA polymerase.

Selectivity at the enzyme level: effects on human DNA polymerases α, β, γ and ε

To gain further insight into the origins of the selective antiviral activity exhibited by emtricitabine, the inhibition of purified human HeLa cell DNA polymerases α, β, γ and ε by the 5'-triphosphate of emtricitabine was examined under steady-state conditions and compared to the inhibition of HIV RT [21]. Activated calf thymus DNA was used as the template for analysis of each enzyme. Under these conditions, emtricitabine 5'-triphosphate was a weak inhibitor of each of the human DNA polymerases when compared to HIV RT. Apparent K_I values were 6.0 μM for polymerase α, 17 μM for polymerase β, 6.0 μM for polymerase γ, and 150 μM for polymerase ε, compared to a K_I value of 0.17 μM for HIV-1 RT.

Long-term treatment with nucleoside analogues has been associated with various forms of toxicity. Inhibition of human DNA polymerase γ (Pol γ) is one of the proposed mechanisms for nucleoside analog-derived toxicity. The 5'-triphosphate form of many nucleoside analogs has been shown to serve as a substrate for Pol γ resulting in an inhibition of mitochondrial DNA synthesis. Therefore the potential for emtricitabine 5'-triphosphate and lamivudine 5'-triphosphate to serve as substrates for Pol γ was investigated (J. Feng, personal communication, Triangle Pharmaceuticals). For dCTP, emtricitabine 5'-triphosphate, and lamivudine 5'-triphosphate, the order of incorporation efficiency is dCTP > lamivudine 5'-triphosphate > emtricitabine 5'-triphosphate. The low rate of incorporation and poor binding affinity of emtricitabine 5'-triphosphate makes it the least favorable substrate for Pol γ.

The excision of 2', 3'-dideoxynucleoside 5'-monophosphate from the 3'-terminus of DNA by Pol γ-associated exonuclease activity can rescue mitochondrial DNA synthesis from the chain terminating effect of a nucleotide analog (J. Feng, personal communication, Triangle Pharmaceuticals). Unmodified primers terminated with the natural cytidine nucleotide (dCMP) have been shown to be the best substrates for the Pol γ-associated exonuclease, followed by emtricitabine 5'-monophosphate and lamivudine 5'-monophosphate. Little difference was observed in the ability of the Pol γ-associated exonuclease to excise emtricitabine 5'-monophosphate and lamivudine 5'-monophosphate from a terminated primer. Overall, these studies with DNA Pol γ demonstrated that emtricitabine has a lower inhibitory effect on the mitochondrial enzyme than does lamivudine, and therefore may be less likely to cause mitochondrial toxicity in the long term.

In vivo antiviral activity

Anti-HIV activity

The anti-HIV activity of emtricitabine has been tested in SCID (severe combined immunodeficient) mice. Mice were reconstituted with human PBMCs [31] and, after two weeks, infected with the $HIV\text{-}1_{A1018}$ virus. Drug therapy was initiated one day before infection. Test compounds were administered intraperitoneally twice daily at 30 mg/kg. Viral inhibition was measured by quantitative coculture of infections HIV-1, and

quantitative RNA viral load measurement on peritoneal wash cells, lymph nodes, spleen cells and plasma. At the concentration used in this study, emtricitabine completely inhibited viral infection.

Black and Furman (personal communication, Triangle Pharmaceuticals) evaluated the anti-HIV activity of orally administered emtricitabine and lamivudine side by side in the HuPBMC-SCID mouse model. Groups of 12 or 15 female C.N-17 SCID mice were reconstituted by the intraperitoneal (ip) injection of 1.3×10^8 human PBMCs. Two weeks later, the mice were infected ip with 2000 tissue culture infectious doses (TCID) of HIV-1_{A018}. After infection, mice were weighed and randomized to treatment or control groups. Mice in the treatment groups received a single ip-loading dose of drug. Drugs were then administered in drinking water, which contained 0.3 mg/ml emtricitabine. Water bottles were weighed at the beginning and the end of treatment, so that water consumption could be calculated. Seven days after infection, mice were anesthestized, weighed, and euthanized by exsanguination. Viral load in plasma was measured using a real-time reverse transcriptase-polymerase chain reaction (RT-PCR). Both emtricitabine and lamivudine were well tolerated during the seven days of the study with no evident toxicity. Average animal weights and daily water consumption for the three groups were similar. Thus average daily doses of the two drugs were similar, about 60 mg/kg. In the control group the geometric mean viral load was 2.5×10^4 copies/ml. Emtricitabine reduced plasma viral loads to below the limit of detection in all 12 treated mice, and lamivudine reduced plasma viral loads to below the limit of detection in 11 of the 12 treated mice. The reductions in viral loads in both treatment groups were statistically significant ($p<10^{-5}$) compared to control, but did not differ significantly from each other.

Anti-hepadnavirus activity

Chimeric mouse model

The *in vivo* anti-hepatitis activity of emtricitabine was first tested in a chimeric mouse model. NIH bg-nu-xid mice were subcutaneously injected with suspensions of 10^7 HepG2 2.2.15 cells. Subcutaneous injection of these cells resulted in the development of tumors of HepG2 2.2.15 cells producing HBV. HBV could be detected in serum samples from the tumor-bearing mice using an immunoaffinity system linked to quantitative PCR.

Beginning one week postinjection, mice were dosed orally with 0.9, 3.5, 18.4 and 88.8 mg/kg/day of emtricitabine. Comparison of tumor progression and human α-fetoprotein levels in control versus drugdosed mice indicated that emtricitabine did not have antitumor activity. However, emtricitabine at the 18.4 and 88.8 mg/kg/day doses did significantly reduce circulatory levels of HBV DNA. Examination of tumor extracts in these two dose groups revealed a marked reduction in intracellular levels of replication DNA HBV intermediates, including double-stranded linear DNA.

Transgenic SCID mice

Anti-HBV activity has also been reported for emtricitabine in HBV transgenic SCID mice [32]. A group of five mice was treated with emtricitabine at 100 mg/kg/day ip for six days, and observed for an additional six days posttreatment. By day three of treatment, two mice had undetectable HBsAg, and an additional two mice cleared HBsAg on day five. The remaining mouse cleared HBsAg on day eight. HBV DNA levels in blood were determined by semiquantitative PCR at days 3, 5, 8 and 12. By day three of treatment, PCR signals had dropped tenfold in all of the mice. By day eight none of the mice had detectable HBV DNA levels by PCR. In contrast to lamivudine, there were no signs of rebound in the levels of HBV DNA during the course of treatment.

DHBV

Duck hepatitis B virus has been used as a surrogate virus to test the *in vivo* activity of compounds under study as potential anti-HBV drugs. Although DHBV is in a different genus of hepadnaviridae than HBV (avihepadnavirus rather than orthohepadnavirus), it has significant enough structural and biological relatedness to HBV to support its use as an *in vivo* model. Emtricitabine was first tested against DHBV in a pilot study in which congenitally infected ducklings were treated orally with a dose of 50 mg/kg bid. This study established that emtricitabine inhibited viral replication and was well tolerated. In a longer-term study utilizing the 50 mg/kg bid. dosing regimen, four adult ducks were dosed for 12 weeks. Determination of the efficacy of emtricitabine was not a primary goal of this study, however the compound was described as being as effective as 2'-CDG, which reduced viremia by tenfold after four days of treatment and to nearly undetectable levels within a few weeks. Liver biopsies conducted two and six weeks into treatment revealed a significant decline in replicative forms of viral DNA, consistent with the ability of emtricitabine 5'-triphosphate to act as an alternative substrate inhibitor of DHBV polymerase. However, levels of cccDNA (circular covalently closed DNA) declined much more slowly, dropping 20%, 40% and 60% after two, six and 12 weeks of therapy, respectively.

WHV

The woodchuck hepatitis virus (WHV) and its natural host, the eastern woodchuck Marmota monax, are the most accepted and most frequently used model of hepatitis-induced disease. This is principally for three reasons: (1) WHV is more similar to HBV than the other hepadnaviridae available in animal model systems. Both HBV and WHV are members of the genus orthohepadnavirus and share approximately 70% nucleotide sequence homology [33]; (2) the range of hepatic injury produced by chronic infections in woodchucks closely resembles that seen in HBV-infected humans; (3) the relative *in vivo* effectiveness of antiviral agents is comparable against WHV and HBV. Four antiviral agents, which had previously been studied for anti-HBV activity in clinical trials, were studied in WHV and the results compared back to the clinical trial

results [34]. This comparison showed parallel activity to that observed in the clinic and reinforced the utility of chronic WHV infection in woodchucks for *in vivo* evaluation of agents being developed for chronic HBV infection.

Emtricitabine has demonstrated profound anti-hepatitis virus activity in the woodchuck model. In an oral dosing study, five groups of chronically infected woodchucks were given emtricitabine QD. at one of five doses: 0.3, 1.0, 3.0, 10 or 30 mg/kg for four weeks [35]. At doses of 3.0 mg/kg and greater, emtricitabine induced a statistically significant reduction in both serum viremia and replicative intermediates. The largest reduction in viremia, approximately 4.9 $\log_{10}$, and in replicative intermediates, approximately 80-fold, was seen at the 30 mg/kg dose. No significant effect on the levels of intrahepatic RNA, serum levels of WHsAg, or the appearance of antibodies to WHsAg or WHcAg in the serum were observed. Viremia returned to pretreatment levels within one to two weeks following the end of treatment at all doses. This rapid rebound is consistent with a lack of significant impact on levels of WHV cccDNA.

These results are very similar to those obtained by the same group in a separate oral QD. dosing study of chronically infected woodchucks with lamivudine. The decreases in serum viremia and replicative intermediates in liver tissue produced by lamivudine are similar to those observed for emtricitabine at comparable exposures of drug. In addition, the rate and degree of viral rebound observed upon cessation of therapy was virtually identical for both compounds.

Cullen et al. have studied the effect of emtricitabine on WHV in naturally infected, wild-caught woodchucks [36]. Animals were dosed ip at either 20 or 30 mg/kg BID for four weeks. Administration of the 20 mg/kg dose suppressed WHV DNA levels from six- to 49-fold (average of 27-fold in the six animal groups). WHV DNA polymerase in serum was reduced in a similar fashion. Serum DNA polymerase activity was measured by the incorporation of [^{32}P] dCTP into WHV DNA. A more profound effect was seen at the 30 mg/kg dose. Serum WHV DNA levels were reduced from 20- to 150-fold (average of 56-fold) in the six animal groups. Serum DNA polymerase activity was similarly reduced. WHV DNA levels in the liver biopsy specimens were also reduced in all six of the animals in the 30 mg/kg-treatment group. Reductions ranged from 68% up to 98% of the pretreatment levels. The authors state that while the level of replicative intermediates remained close to those seen pretreatment, the WHV genome was being shifted toward shorter fragments. In this study, as in the study of Korba et al., levels of WHsAg or antibodies against WHsAg or WHcAg did not change [35].

In vitro resistance

HIV resistance

Because the emergence of drug-resistant virus is a major concern in anti-HIV chemotherapy and helps define the combinations within which a drug will be useful, the development of resistance to emtricitabine was examined by passaging virus *in vitro* in the presence of drug. In a study reported by Tisdale et al., the HXB2 strain of HIV-1 or the zidovudine-resistant mutant, HIV-1_{RTMC} (contains D67N, K70R, T215Y, and K219Q

mutations), was passaged in MT4 cells in the presence of increasing concentrations of emtricitabine or lamivudine [11]. Rapid emergence of resistance occurred with both compounds. By the fourth passage of HXB2 and the second passage of HIV-1_{RTMC}, EC_{50} values exceeded 50 μM and by passage six, EC_{50} values were in excess of 250 μM. These variants were highly cross-resistant to lamivudine and emtricitabine, but showed no cross-resistance to zidovudine, didanosine, or nevirapine. DNA sequence analysis showed a change at codon 184, with methionine replaced by valine. Based on synergy data with zidovudine and the lack of cross-resistance, the authors suggested that combination of the oxathiolane analogues with zidovudine might slow the emergence of resistance to emtricitabine and/or lamivudine. Indeed, passaging virus in the presence of increasing concentrations of emtricitabine and 50 nM zidovudine was able to delay appreciably, but not prevent, the emergence of emtricitabine-resistant virus [11].

In experiments performed by Schinazi et al., the potential for HIV-1 resistance to develop to lamivudine and emtricitabine was evaluated by serial passage of the virus in human PBMCs in the presence of increasing drug concentrations [37]. Results presented in Figure 3 show that after two weeks of infection, 0.1 μM lamivudine was no longer able to inhibit virus replication, and drug-resistant variants dominated the replicating virus population. In contrast, using identical conditions, emtricitabine remained highly active, reducing virus replication by 80%. Emtricitabine was still able to inhibit virus replication by 50% at week three. At week four the concentration of lamivudine was increased 100-fold to 10 μM, and the concentration of emtricitabine was increased tenfold to 1 μM to produce the same level of inhibition. Emtricitabine retained up to a tenfold potency advantage over lamivudine after four weeks of passaging. These data in primary human cells suggest that emtricitabine may delay the breakthrough of resistant viruses relative to lamivudine.

DNA sequence analysis of the reverse transcriptase gene amplified from resistant viruses generated in these passaging experiments consistently identified mutations at codon 184, where methionine was changed to either valine or isoleucine. Resistant variants were cross-resistant to both emtricitabine and lamivudine, but remained sensitive to zalcitabine, didanosine, zidovudine, PFA, 3'-fluoro-3'-deoxythymidine (FLT), and two non-nucleoside reverse transcriptase inhibitors, the TIBO compound R82150 and the bis(heteroaryl) piperazine derivative U-87201E [37].

Biochemical studies were performed to quantify the change in susceptibility of HIV RT derived from virus resistant to emtricitabine 5'-triphosphate, lamivudine 5'-triphosphate, and the zalcitabine 5'-triphosphate [37]. Virus particle-derived RT was obtained from the supernatant of human PBMCs that were infected with M184V mutant virus. The mutated enzyme was 15-fold less sensitive to inhibition by emtricitabine 5'-triphosphate or lamivudine 5'-triphosphate than was wild type HIV_{LAI}-RT. However, only a threefold decrease in susceptibility was noted for zalcitabine 5'-triphosphate. Similar results were reported using a highly purified cloned RT containing the M184V mutation [27]. In these studies the K_i values for emtricitabine 5'-triphosphate and lamivudine 5'-triphosphate were increased 320- and 80-fold, respectively, compared to wild type HIV RT. Wilson et al. [38], using steady state and pre-steady-state kinetic analysis, examined the effect of the M184V mutation on HIV-1 RT catalytic function. These kinetic studies showed that the M184V mutation did not

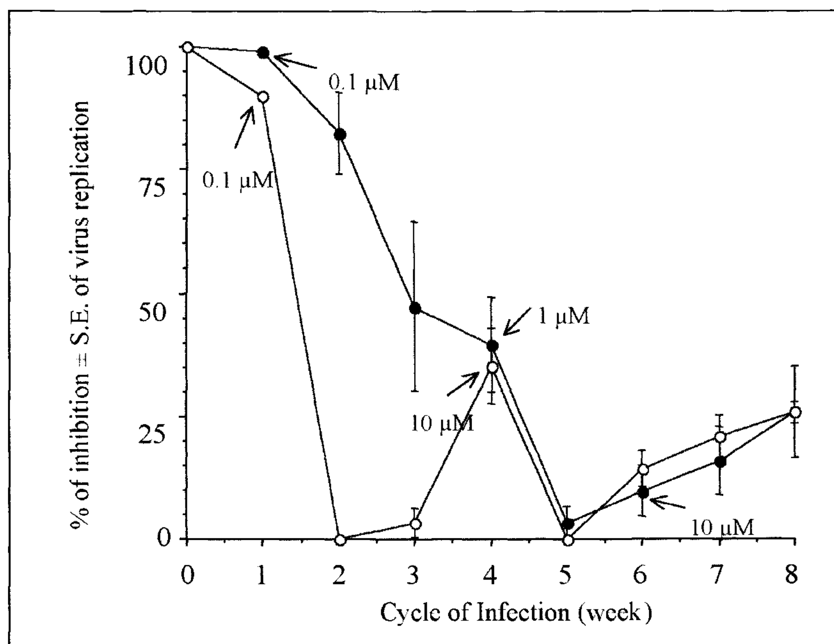

Figure 3. HIV-1 breakthrough in the presence of emtricitabine (●) and lamivudine (○) [37].

alter either the K_m or the k_{cat} values for the natural substrates, but did cause a 300-fold increase in the K_i for emtricitabine 5'-triphosphate.

The clinical emergence of the M184V virus was first reported by Schinazi et al. in a patient who had received lamivudine [37]. The M184V mutation was also found in recombinant virus prepared using RNA isolated from the plasma of three patients who had received emtricitabine monotherapy for two weeks. Phenotypic analysis in MT-2 cells demonstrated all three of these viruses to be resistant to emtricitabine and cross-resistant to lamivudine. The EC_{50} values of the recombinant virus for both emtricitabine and lamivudine increased greater than 50-fold relative to the EC_{50} value against wild type HIV-1_{LAI}. One of these isolates contained the M41L and T215Y mutations, but remained sensitive to inhibition by zidovudine. This observation is in keeping with a report by Schinazi et al. that the introduction of the M184V mutation against a zidovudine-resistant background can result in increased sensitivity to zidovudine [37].

Emtricitabine activity has been evaluated extensively against a panel of clinical isolates (Table 9). The panel consists of a series of recombinant wild type clinical isolates and 35 recombinant isolates containing anywhere from one to 12 mutations. Consistent with earlier passaging experiments, a high level of resistance is imparted by introduction of the M184V mutation against any mutation background. Moderate resistance to emtricitabine is seen for the highly mutated isolate (M41L, E44D, D67N, T69D, L74I, K101E, V108I, V118I, Y181C, G190A, L210W, T215Y) that contains the two mutations associated with moderate resistance to lamivudine (E44D, V118I).

Table 9. Phenotypic analysis of recombinant viruses generated from clinical isolates. analysis of emtricitabine resistance profile

Genotype	EC_{50} (μM)[a]		
	Emtricitabine	Lamivudine	Zidovudine
WT: LAI	0.619[b]	2.567[b]	0.487[b]
WT (n=16)	0.64[c]	2.998[c]	0.917[c]
L100I (A)	0.176	2.125	0.037
L100I (B)	0.595	2.350	0.160
G190A	0.220	0.900	0.205
G333E	1.350	1.265	0.260
M184V	>20.00	>50.00	0.140
K103T	0.330	1.100	0.140
V108I	0.205	0.650	0.135
K103N (A)	0.825	3.150	0.595
K103N (B)	0.680	4.000	0.250
K103N, M184V (A)	>20.00	>50.00	0.185
K103N, M184V (B)	>20.00	>20.00	3.02
E138K, M184V (A)	>20.00	>50.00	0.145
E138K, M184V (B)	>20.00	>50.00	0.130
E13Q, M184V	>20.00	>50.00	0.890
A98S, M184V	>20.00	>50.00	0.120
L74V, K103N	2.34	>2.26	1.87
K101Q, E138K	1.38	1.94	0.54
K103R, Y188C	1.13	1.16	0.72
K103N, Y181C	0.55	1.17	0.29
K70R, L74V, M184V	>20.00	>20.00	0.90
K103T, V106I, M184V	>20.00	>20.00	0.36
K101Q, E138K, K103N	0.55	0.48	0.67
K103N, V108I, M184V	>20.00	>20.00	0.31
T215Y, K103N, L210W	0.73	1.05	>2.00
M41L, K101R, M184V, T215Y	>20.00	>20.00	>2.00
A98S, F116Y, Q151M, T215Y	1.45	0.55	>2.00
T69N, K70R, M184V, K219Q	>20.00	>20.00	1.23
D67N, K70R, M184V, G190A	>20.00	>50.00	0.35
D67N, T69D, K103R, T219Q	2.58	2.92	5.75
A62V, A98S, K101D, K102Q, M184V	>20.00	>50.00	0.35
M41L, D67N, M184V, L210W, T215Y	>20.00	>20.00	>2.00
D67N, K70R, E138A, M184V, T215Y, K219E	>20.00	>20.00	>2.00
M41L, D67N, Y181C, M184V, L210W, T215Y	>25.00	>20.00	>2.00
M41L, D67N, T69D, V108I, M184V, T215Y	>20.00	>20.00	>2.00
A62V, V75M, K103N, F116Y, Q151M, M184V	>20.00	>20.00	>2.00
D67N, T69D, K70R, K103N, M184V, T215Y, K219Q	>20.00	>20.00	>2.00

Table 9. Continued

Genotype	EC_{50} (μM)[a]		
	Emtricitabine	Lamivudine	Zidovudine
M41L, D67N, A98G, K101E, K103N, M184V, G190A, L210W, T215Y, G333E	>20.00	>20.00	>2.00
M41L, E44D, D67N, T69D, L74I, K101E, V108I, V118I, Y181C, G190A, L210W, T215Y	7.27	6.63	1.85

[a] EC_{50} values are expressed as the median value of at least three replicates, unless otherwise noted.
[b] EC_{50} values is the average value of at least 10 replicates.
[c] EC_{50} values are the average of replicates from 16 different recombinants displaying a WT genotype.

HBV Resistance

Treatment of HBV-infected patients with lamivudine has been shown to be effective in suppressing virus replication and in reducing inflammatory activity. However, resistance to this agent has been documented that is associated with mutations in the YMDD motif in domain C of the viral DNA polymerase, analogous to changes seen in the YMDD motif of HIV-RT. Methionine 204 [39] had mutated to either isoleucine (M204I) or valine (M204V). The M204V mutation is almost always observed in conjunction with an additional mutation, L180M in the B domain. Because of the sequence homology between the active sites of HIV RT and the HBV DNA polymerase [29], the importance of the YMDD motif to the catalytic activity of both polymerases, and the role of the methionine in the resistance of HIV-1 to lamivudine and emtricitabine, it would be anticipated that HBV containing either the M204I or the M204V mutation is cross-resistant to emtricitabine. Inhibition assays performed using HepAD38 and HepAD79 cells, which replicate wild type and the M204V mutant HBV, respectively [40, 41], confirmed that the methionine-to-valine mutation conferred resistance to emtricitabine as well as to lamivudine. EC_{50} values for lamivudine and emtricitabine versus wild type and mutant HBV were 0.09 and 1.3 μM, and 0.04 and 1.25 μM, respectively. Interestingly, introduction of the M204V mutation into the HBV polymerase did not impart the same degree of resistance to emtricitabine or lamivudine seen upon introduction of the M184V mutation into the HIV polymerase.

Zoulim et al. (personal communication from F. Zoulim, INSERM, France) using a duck (DHBV) polymerase assay reported that mutations conferring resistance to lamivudine showed cross-resistance to emtricitabine. In their studies, mutations were made in the DHBV DNA polymerase at M204 and L180 that correspond to the M204 and the L180 mutations in HBV. The inhibitory activity of the 5'-triphosphates of

lamivudine and emtricitabine was compared against wild type and mutant polymerases using enzyme expressed in a coupled transcription/translation rabbit reticulocyte lysate system. Both lamivudine 5'-triphosphate and emtricitabine 5'-triphosphate were inhibitors of viral minus-strand DNA synthesis, with IC_{50} values of 6.1 ± 3.5 μM and 8.5 ± 4.1 μM, respectively. When tested against the M204V-, M204I-, and M204V + L180M- containing mutants, the IC_{50} values for lamivudine 5'-triphosphate and emtricitabine 5'-triphosphate increased markedly (>100 μM). In cell culture assays using LMH cells transiently transfected with DHBV genomes containing mutations M204V, M204I, or M204V + L180M, neither lamivudine nor emtricitabine showed any antiviral activity.

Toxicological studies

Cytotoxicity

The cytotoxicity of emtricitabine has been evaluated extensively *in vitro* (Table 10). In all of the cell lines examined, cell growth was not affected at concentrations of emtricitabine up to and including 200 mM [7]. Similar results were obtained with lamivudine [8, 13, 42, 43].

Because of the apparent correlation between toxicity to bone marrow progenitor cells *in vitro* and bone marrow suppression *in vivo*, human bone marrow progenitor colony forming assays were performed. The concentration of emtricitabine required to inhibit the formation of granulocyte-macrophage (GFU-GM) colonies by 50% (CC_{50}) was 300 ± 40 μM (n = 6). The CC_{50} for erythroid colonies (BFU-E) was 220 ± 8 μM (n = 6). CC_{50} values for lamivudine are comparable with a value for GFU-GM of 260 ± 8 μM and a value for BFU-E of 180 ± 2 μM [8, 14, 42]. CC_{50} values of the (+) isomer of 3TC in the bone marrow progenitor cell assay were determined to be 10 ± 2 μM for GFU-GM and 4 ± 1 μM for BFU-E. It is interesting that the (+)-isomer of lamivudine shows bone marrow toxicity, while the (+)-isomer of emtricitabine has CC_{50} values comparable to those of the (-)-isomer.

Although the mechanism(s) responsible for the toxic side effects of the nucleoside antiviral agents is multifactorial, delayed mitochondrial toxicity is believed to be a major underlying contributor [44]. Previous studies on ddC-induced peripheral neuropathy [45] and AZT-induced myopathy [46] have revealed a depletion of mitochondrial DNA content in drug-treated cells. This depletion could account for toxicities observed in the clinic. In an effort to evaluate the potential for mitochondrial toxicity, human hepatoblastoma HepG2 cells were incubated with emtricitabine at concentrations ranging between 0.1 and 10 μM for two weeks [47] and up to 100 μM for up to eight weeks (J. Jeffrey, personal communication, Triangle Pharmaceuticals). Under these conditions, emtricitabine had no adverse effects on cell growth, mitochondrial DNA synthesis or lactic acid production. In a separate study conducted in HepG2 cells exposed to concentrations of emtricitabine ranging from 0.1 to 10 μM for eight days, no effects on mitochondrial morphology were observed by transmission electron microscopy.

Table 10. Cytotoxicity of emtricitabine in comparison to lamivudine and zidovudine

	CC_{50} (μM)		
Cells	emtricitabine	lamivudine	zidovudine
MT4	>100[a], >200[b]	>100[a], >33[b]	20[a], > 100[b]
PBMC	>100[a]	>100[a]	>100[a]
CEM	>100[a], >100[b]	>100[a], >100[b]	14.3[a], >6[b]
Vero	>100[a]	>100[a]	28.0[a]
IM9	>100[b]	>100[b]	70[b]
Molt 4	>100[b]	>100[b]	10[b]
HepG22.2.15	>200[b], >200[c]	>200[b]	>200[b]

a. [13]
b. [42]
c. [14]

In vivo toxicology

The preclinical toxicology profile of emtricitabine is extremely favorable [48]. In six-month mouse and one-year monkey studies, CD-1 mice were dosed orally up to 3000 mg/kg/day, and cynomolgus monkeys were dosed orally up to 500 mg/kg/day. Mild, reversible anemia was the only observed toxicity. Anemia occurred only at the highest doses tested, where plasma exposures ($AUC_{0\text{-}24}$) in mice were approximately 150-fold human exposures at the proposed dose of 200 mg/day, and in monkeys, where plasma exposures exceeded 25-fold human exposures. In accord with *in vivo* studies, mitochondria in liver, heart and skeletal muscle examined by transmission election microscopy were normal in mice given oral doses of emtricitabine at 3000 mg/kg/day for six months.

Emtricitabine had no effect on reproduction or development at exposures approximately 100 times human exposure. Reproductive toxicity studies consisted of fertility studies in CD-1 mice and in CD male rats. Developmental toxicology studies were conducted in pregnant mice and in pregnant New Zealand white rabbits.

Genetic toxicology studies consisting of an Ames assay, a mouse lymphoma assay and an assay using Chinese hamster ovary (CHO) cells to detect chromosomal aberrations were all negative.

Preclinical pharmacokinetics, metabolism and excretion

The pharmacokinetics, metabolism and excretion of emtricitabine have been investigated in mice, rats and cynomolgus monkeys after single and repeated dosing [49, 50]. Emtricitabine is rapidly and well absorbed after oral administration in all three species, with oral bioavailability ranging from 58% to 97%. Peak plasma emtricitabine concentrations occur between 0.5 to 2.5 hours post dose. Plasma concentrations of emtricitabine expressed as AUC increase linearly with dose over a wide dose range (up to 1500 mg/kg/day in mice and 500 mg/kg/day in monkeys). Plasma emtricitabine is eliminated with a half-life ($t_{1/2}$) of one to four hours and a total body clearance of ~1.5 L/kg/hr in rats and ~ 0.7 L/kg/hr in monkeys.

Emtricitabine has a volume of distribution (Vd_{ss}, 0.8 – 1.5 L/kg) slightly larger than the total body water in all species, suggesting that it is distributed into both the intracellular and extracellular fluid spaces. The fraction of protein-bound emtricitabine in plasma is <4% for all species.

Tissue distribution studies of ^{14}C-emtricitabine in rats and monkeys confirm that emtricitabine is widely distributed to all tissues [51]. The ^{14}C-radioactivity concentrations in tissues generally decline in parallel with those in plasma, with no indication of drug accumulation. Virtually no radioactivity remains in the body at 72 hours post-dosing. The ^{14}C concentrations in the kidneys, gut and liver exceed those in plasma, while concentrations in CNS tissues were <10% of those in plasma. Renal excretion of unchanged drug is the principal route of emtricitabine elimination. In rats, 91% of the ^{14}C-emtricitabine dose was recovered in the urine after an IV dose, and 74% after an oral dose. Approximately 67% and 41% of an oral ^{14}C dose was recovered in the urine of mice and monkeys, respectively. The majority of the dose recovered in the feces after an oral dose most likely represents unabsorbed drug. Renal clearance of emtricitabine generally exceeded the glomerular filtration rate, suggesting that emtricitabine is excreted by renal tubules. In all three species, metabolism is a minor route of emtricitabine elimination. Over 90% (mice) and 64% (monkeys) of radioactivity recovered in urine was unchanged drug. The principal metabolites, the isomeric 3'-sulfoxides, accounted for ~2% of the dose in mice, 2.6% in rats and 8% in monkeys. The only other metabolite detectable in monkey urine was a 2'-glucuronide metabolite, which accounted for 1.1% of the dose. In mice, very minor metabolites, the 2'-glucuronide, 5'-fluorouridine (all <1-2% of dose) were detected. No 5'-flurouracil was detected in any species.

Clinical development — HIV

Clinical pharmacology (human pharmacokinetic) phase I/II studies

The PK and disposition of emtricitabine following single- or multiple-dose administration have been extensively characterized. Emtricitabine is rapidly and well absorbed following oral administration, with steady-state plasma concentrations reaching levels several-fold above the mean *in vitro* EC_{90} value for anti-HIV activity (Figure 4). At 24 hours post-dose, plasma trough concentrations following a 200 mg QD dose exceeded

the mean *in vitro* EC_{90} for anti-HIV activity by about fourfold. Based on extrapolation of this data, plasma emtricitabine concentrations are expected to be within the range of the EC_{90} at 48 hours post-dose, and within the range of EC_{50} at 72 hours post-dose.

Emtricitabine disposition follows linear kinetics with small inter-subject variability, predictable steady-state concentrations and dose proportionality over a wide dose range. The high concentrations and long half-lives of emtricitabine in plasma (8-9 hours) and emtricitabine-5'-triphosphate (>39 hours) in peripheral blood mononuclear cells (PBMCs) support once daily dosing. Renal excretion of unchanged drug is the principal route of emtricitabine elimination from plasma, and thus presents little potential for metabolic drug interactions. Plasma AUC and total body clearance appear to be correlated with creatinine clearance. A pediatric dose of 6 mg/kg QD has been identified and is under evaluation in phase II studies.

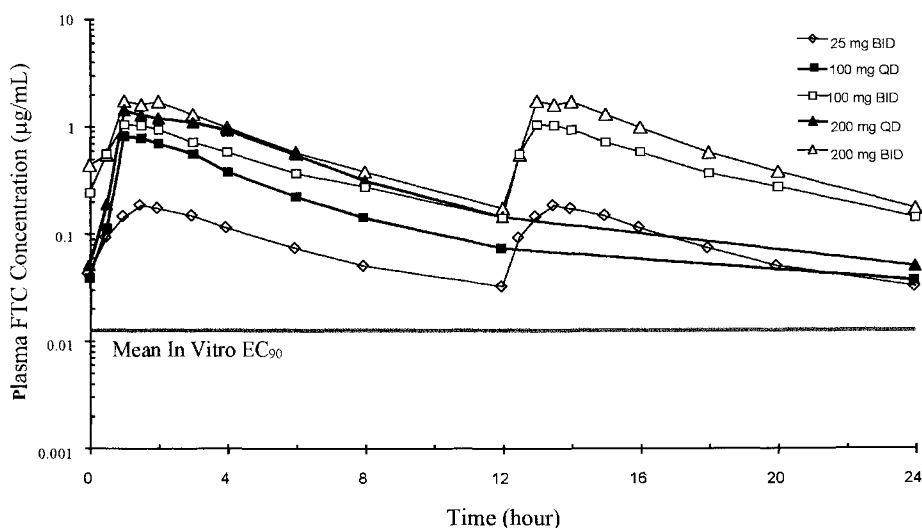

Figure 4. Mean steady-state plasma emtricitabine concentrations following a 25 mg, 100 mg QD, 100 mg BID, 200 mg QD or 200 mg BID dosing regimen.

Drug interaction studies

The PK of emtricitabine was not significantly affected when it was coadministered with either zidovudine (ZDV) or stavudine (d4T). A single dose of emtricitabine had no clinically significant PK effect on d4T, but did increase the AUC and C_{max} of ZDV by 26% and 66%, respectively. Due to large intersubject variability and the small sample size (n = 6), the actual extent of interaction may be less than that reported in this study.

In addition, the moderate increase in ZDV exposure observed would not be expected to be clinically significant. No clinically significant PK interactions between emtricitabine and the protease inhibitor indinavir, or the non-nucleoside reverse transcriptase inhibitor emivirine were observed following a single oral administration of emtricitabine to 12 subjects. In a study of nine treatment-naïve, HIV-infected patients enrolled in the ANRS091 trial, the simultaneous administration of FTC, ddI and efavirenz did not significantly affect the PK of any of these drugs.

Because emtricitabine is primarily eliminated as unchanged drug in urine, a study focusing on the potential interactions between emtricitabine and famciclovir at the site of urinary excretion was conducted. Famciclovir was chosen as a typical drug for this evaluation because its active form in plasma (penciclovir) is primarily eliminated in urine. No interactions were seen.

Dose selection trials

Two short-term monotherapy trials were conducted to define a dosage regimen for use in Phase III therapeutic trials, FTC-101 and FTC-102. Protocol FTC-101 was an open-label, sequential, dose-ranging trial evaluating the *in vivo* antiviral activity of emtricitabine in HIV-infected patients given 14 days of monotherapy at 25 mg BID, 100 mg QD, 100 mg BID, 200 mg QD, and 200 mg BID. A total of 41 patients (N = 8 or 9 per dose group) naïve to lamivudine and abacavir were enrolled. At screening, $CD4^+$ cell count ranged from 198 to 1071 cells/mm^3 and plasma HIV-1 RNA ranged from 3.9 to 5.9 log_{10} copies/mL. Plasma HIV-1 RNA was measured at baseline and frequently over the 14 days of treatment. The pharmacokinetics of emtricitabine in plasma and emtricitabine 5'-triphosphate levels in PBMCs were also evaluated.

Potent antiretroviral activity occurred in all dosage cohorts, with a strong trend towards greater activity at the higher doses (Figure 5). Viral suppression in the 200 mg QD group was comparable to that observed in the 200 mg BID group. The 200 mg QD dose group showed a median drop from baseline at day 15 of 1.9 log_{10}, as compared to 1.3, 1.5, 1.7, and 1.9 log_{10} for the 25 mg BID, 100 mg QD, 100 mg BID, and 200 mg BID dose groups, respectively. The onset of anti-HIV activity occurred within 48 hours of initiating emtricitabine dosing with the most rapid viral load decline occurring between days 3 and 8. Results of statistical analyses of HIV-1 RNA AAUCMB (average area under the curve minus baseline), change from baseline at day 15 (last day on study treatment), and maximum change from baseline, consistently supported the dose-response relationship and the maximal antiviral effect at the 200 mg QD and 200 mg BID doses.

Protocol FTC-102 was an open-label, randomized, parallel-group monotherapy trial comparing three once-daily dosage regimens of emtricitabine (25 mg, 100 mg and 200 mg QD) and the approved lamivudine regimen (150 mg BID) during 10 days of monotherapy. A total of 81 patients, naïve to lamivudine and abacavir, were randomized to one of the four treatment regimens. At screening, median $CD4^+$ cell count ranged from 350 to 431 cells/mm^3, and median plasma HIV-1 RNA ranged from 4.3 to 4.7 log_{10} copies/mL. Plasma HIV-1 RNA levels were measured at baseline and frequently over the 10 days of treatment.

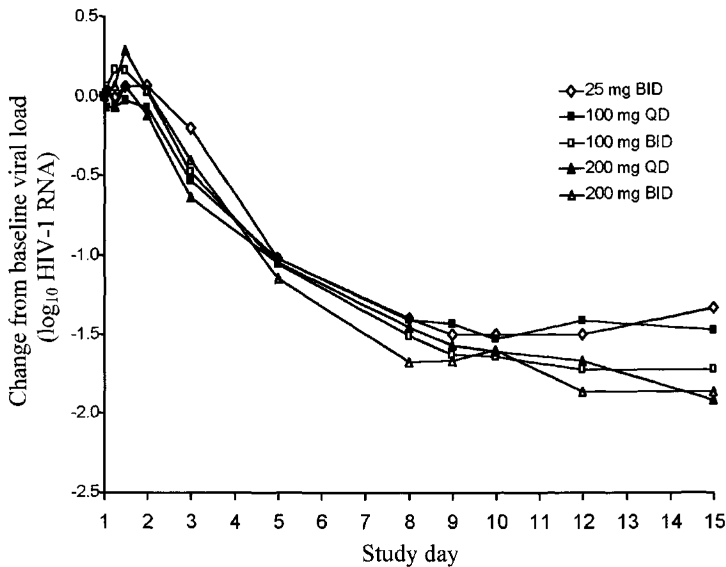

Figure 5. Study FTC-101: Median change in HIV-1 RNA from baseline.

This randomized, controlled study confirmed the dose-response results of FTC-101, with the most potent antiviral activity occurring in the emtricitabine 200 mg QD dose group (Figure 6). Median change from baseline at day 11 was 1.50, 1.58, and 1.69 log_{10} for the 25, 100 and 200 mg QD emtricitabine doses, respectively, and was 1.48 log_{10} for the 150 mg BID lamivudine dose. Results from statistical analyses of HIV-1 RNA AAUCMB, change from baseline at day 11 (last day on study treatment), and maximum change from baseline consistently distinguish 200 mg QD from the lower emtricitabine doses.

In addition to the greater activity of emtricitabine 200 mg QD based on change from baseline in HIV-1 RNA, there was also a greater proportion of patients who achieved the limit of assay detection (400 copies/mL) or who had a two log_{10} decrease from baseline on the FTC 200 mg QD dose (Figure 7).

The dose-response relationship was further evaluated by correlating HIV-RNA AAUCMB with emtricitabine daily dose using a pharmacological E_{max} model, $E = (E_{max} \cdot Dose)/(EC_{50} + Dose)$. The pharmacological dose-response curves show that the effect of emtricitabine on HIV-1 RNA suppression had reached the maximal effect at doses > 200 mg per day. Very little additional effect on HIV-1 RNA suppression was observed with increasing the dose from 200 mg to 400 mg per day.

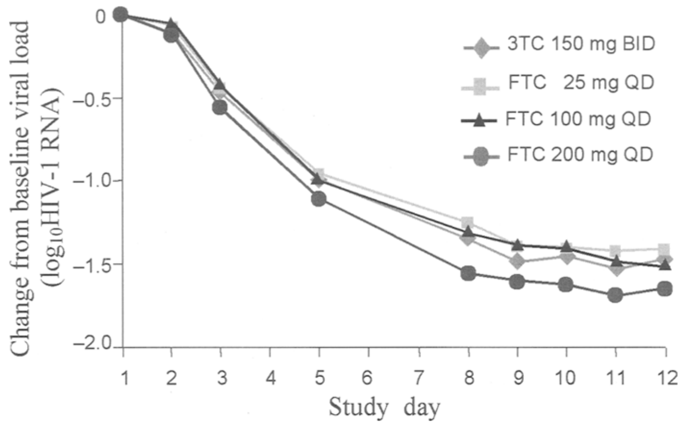

Figure 6. FTC-102 mean change from baseline in plasma HIV-1 RNA.

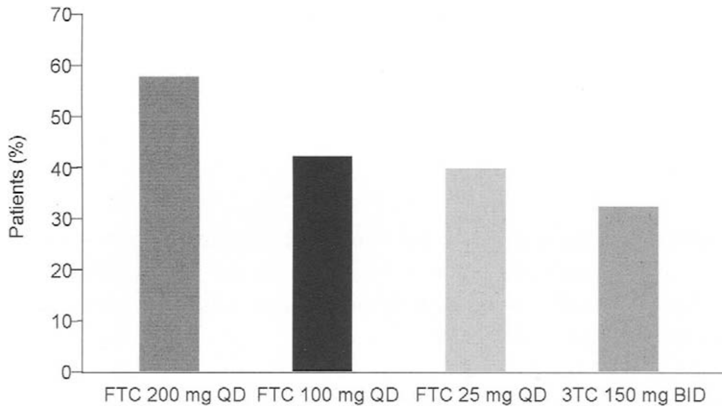

Figure 7. FTC-102: Proportion of patients with HIV-1 RNA < 400 copies/mL or >2 log_{10} decrease from baseline.

The steady-state plasma and intracellular pharmacokinetics of emtricitabine support the selection of a 200 mg QD dose. The steady-state intracellular emtricitabine 5'-triphosphate concentrations increased in a dose-related fashion, reaching an apparent plateau level at emtricitabine daily doses of 200 mg or greater. The clinical antiviral activity correlates well with the intracellular 5'-triphosphate levels in PBMCs (Figure 8). As the emtricitabine dose increased, intracellular emtricitabine 5'-triphosphate levels and viral load suppression increased, reaching an apparent plateau at daily doses of ≥200 mg.

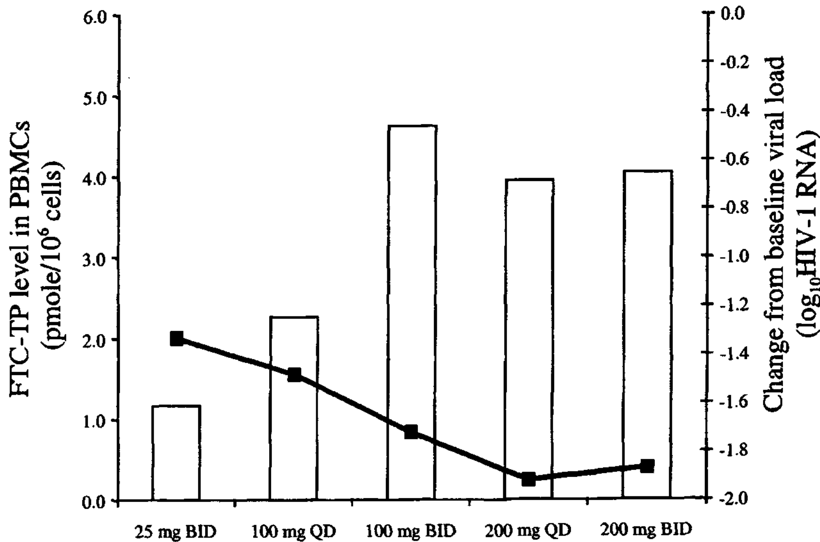

Figure 8. FTC-101: Correlation of antiviral activity of FTC with emtricitabine 5'-triphosphate levels of PBMCs.

Phase II/III clinical studies

A series of phase II/III clinical studies have been conducted with emtricitabine. An overview of each study is given below.

FTC-201: A Pilot Study Evaluating the Efficacy and Tolerance of the Combination Emtricitabine + ddI + efavirenz Administered Once Daily in the Treatment of HIV-1-Infected, Antiretroviral-Naïve Patients (ANRS091-Montana Study).

This Phase II study is being conducted by the ANRS (Agence Nationale de la Recherche sur le SIDA, Paris France) to evaluate a once-daily regimen of FTC + ddI + efavirenz. To date, 34 of 40 treatment-naive patients have completed 96 weeks. Results demonstrate durable antiviral and immunologic effects lasting for the 96-week period using a fully once-a-day regimen. Using a non-completer-equals-failure analysis at 96 weeks, 85% and 80% of patients maintained a plasma HIV RNA level below 400 and 50 copies/mL, respectively. There was a median $CD4^+$ cell count rise of 259 cells/μL at week 96. Overall, the regimen has been well tolerated. The most common treatment-related adverse events occurred during the first 24 weeks of the study, and were mild to moderate in severity.

FTC-303: A Randomized, Open-Label Equivalence Study of Emtricitabine Versus Lamivudine in Patients on a Stable Triple Antiretroviral Therapy Regimen Containing Lamivudine Plus Stavudine or Zidovudine, and a Protease Inhibitor or a Non-Nucleoside Reverse Transcriptase Inhibitor.

Study FTC-303 was a randomized (2:1), open-label, 48-week equivalence study of FTC vs. 3TC in 440 patients on a stable (HIV RNA ≤ 400 copies/mL) triple therapy regimen (≥12 weeks) containing 3TC. Patients were randomized to continue 3TC or switch to FTC by screening viral load (≤50 and >50-400 copies/mL) and by the presence of a protease inhibitor or non-nucleoside reverse transcriptase inhibitor in their regimens. At entry, the treatment groups were comparable with regard to race, gender, age, and baseline disease. Patients had a mean age of 41.5 (range 22-80) years, 64% were Caucasian, and 86% male. Median baseline $CD4^+$ cell count was 488 (range 37-1909) cells/mm^3 and median plasma HIV-1 RNA was 50 copies/mL. The median duration of previous antiretroviral therapy was 28 months.

Overall, 79% of patients completed the 48-week study. The reasons for early termination from the study were similar between treatment groups. Thirteen (4%) patients in the FTC group discontinued the study early due to an adverse event, while one patient (1%) died due to a heroin overdose in the 3TC group. Through 48 weeks of therapy, the proportion of patients who had confirmed virological loss of response was 7.8% in the FTC arm and 7.5% in the 3TC arm. These findings support the conclusion of equivalent safety and virologic efficacy of 200 mg once daily FTC compared to 150 mg 3TC administered twice daily.

FTC-302: A Randomized, Double-Blind Equivalence Trial Comparing Emtricitabine to Lamivudine within a Triple Combination Regimen in Antiretroviral-Drug-Naïve, HIV-1 Infected Patients.

Study FTC-302 was a randomized, double-blind equivalence study of FTC versus 3TC in combination with stavudine and either nevirapine (patients with HIV-1 RNA ≥100,000 copies/mL) or efavirenz (patients with HIV-1 RNA > 100,000 copies/mL) in 468 anti-retroviral-naïve patients. Of the 468 patients treated, 385 (82%) received FTC/3TC plus stavudine and nevirapine, while 83 (18%) received FTC/3TC plus stavudine and efavirenz. Seventy-four percent (74%) of the patients completed 48 weeks of therapy. The treatment groups were comparable with regard to race, gender, age, and baseline characteristics. Median age at entry was 32 years (range 18-63), 77% were black African, and 59% were female. Median screening $CD4^+$ cell count was 373 cells/mm^3 (range 140-1455), and median plasma HIV-1 RNA was 4.6 $\log_{10}$ copies/mL.

The majority of patients in both treatment arms achieved and maintained a plasma HIV-1 RNA ≤ 400 copies/mL through 48 weeks (65% receiving FTC and 71% receiving 3TC), with 60% (FTC) and 64% (3TC) of patients having a plasma HIV-1 RNA < 50 copies/mL at week 48.

Thirty-three FTC-treated patients (14%) and 23 3TC-treated patients (10%) were confirmed virological failures during the study. Interestingly, more of the FTC-treated

virologic failures (13/33, 39%) had no genotypic changes associated with the medications used in the triple therapy regimen, compared to only three (18%) of the 3TC-treated patients. Seven (21%) and 11 (48%) of the FTC- and the 3TC-treated patients, respectively, had the M184V mutation at time of failure. The immunologic benefit observed through week 48 was comparable in each treatment arm, with a mean increase from baseline in absolute $CD4^+$ cell count of approximately 200 cells/mm^3 and a 10% increase in CD4%.

Eleven percent of the patients in each treatment arm discontinued blinded study medication due to an adverse event during the study, with the majority of these discontinuations associated with hepatotoxicities attributed to the use of nevirapine in the triple therapy regimen.

These results confirm that patients in both treatment arms of study FTC-302 derived significant virologic and immunologic benefit during the trial. Results from this trial compare favorably to other randomized, well-controlled, international clinical trials using standard-of-care triple therapy regimens and support the conclusion of equivalent efficacy and safety of FTC with 3TC [52].

Clinical development – HBV

FTCB-101 was a pilot dose-selection study designed to examine the pharmacokinetics and activity of emtricitabine for the treatment of chronic HBV infection [53]. Five once-daily doses of emtricitabine (25 mg, 50 mg, 100 mg, 200 mg and 300 mg) were evaluated sequentially for eight weeks in cohorts each consisting of at least eight patients with chronic HBV infection. Patients were positive for HBsAg and HBV DNA, and naïve to nucleoside analog therapy.

Pharmacokinetic analysis showed emtricitabine to be well absorbed after oral administration, with plasma concentrations reaching levels above the EC_{90} at all doses with once-daily administration. The elimination half-life of emtricitabine from plasma ranged from six to 10 hours, and the steady-state plasma concentrations increased nearly dose proportionally over the 25 mg to 300 mg dose range.

Suppression of HBV DNA depended on baseline viral load and assay sensitivity. On completion of dosing (day 56), HBV DNA suppression reached undetectable levels when assayed by the Digene Hybrid Capture Assay (lower limit of detection 145,000 copies/ml) at low and moderate baseline viral loads and showed a dose effect at high baseline viral load. Using the Chiron Amplicor® HBV Monitor assay (lower limit of detection 400 copies/ml), HBV suppression of greater than 4.0 $\log_{10}$ was observed. The absolute interpretation of dose response was complicated in this study by differing viral loads at baseline between cohorts and by sequential enrollment.

Consistent with what has been reported in HIV trials, emtricitabine was well tolerated. There were no serious adverse events, and no patient was intolerant to the drug. The only observed adverse event of grade II-III severity was headache, which occurred in more than 10% of patients (11% overall).

FTC-102 is a double-blind, randomized trial to evaluate three doses of emtricitabine, 25 mg, 100 mg and 200 mg qd [22, 54]. A total of 98 patients were enrolled, 32 in the

25 mg dose cohort and 33 each in the 100 and 200 mg dose cohorts. The mean age of the patients was 37 years; 70% were male and 88% were of Asian ethnicity. There were no important demographic differences among dose cohorts. The median baseline viral load ($\log_{10}$ c/ml) was 7.57, 7.68 and 7.42 in the 25 mg qd, 100 mg qd and 200 mg qd cohorts, respectively. The initial treatment period was set at 24 weeks, but was extended in a blinded fashion through 48 weeks.

Emtricitabine produced potent inhibition of HBV DNA in a dose-dependent manner. The median change from baseline in HBV DNA for the 25, 100 and 200 mg qd dose groups was 1.7, 3.1 and 3.2 $\log_{10}$, respectively. A similar dose response was seen in suppression of HBV DNA when analyzed by AAUCMB. However, differences between doses were pronounced when HBV suppression was viewed as the percentage of patients in whom viral load falls below the limit of detection (LOD). The percent of patients with HBV DNA below the LOD (4700/CmL) was 38% in the 25 mg qd dose cohort, 42% in the 100 mg qd dose cohort and 61% in the 200 mg qd dose cohort.

Based on this data the 200 mg qd dose was selected for evaluation in pivotal therapeutic trials, FTCB-301. FTCB-301 is a double-blind, randomized, placebo-controlled pivotal study designed to prove the safety and efficacy of the emtricitabine 200 mg qd dose for the treatment of chronic HBV infection.

Epilogue

It is apparent that emtricitabine represents one of the most potent anti-HIV agents identified to date, producing an almost two $\log_{10}$ drop in viral load as monotherapy at a 200 mg qd dose. In addition, the clinical profile of emtricitabine has demonstrated the following key features: 1) a plasma half-life of approximately eight to 10 hours with linear kinetics, 2) an intracellular emtricitabine 5'-triphosphate half-life of greater than 39 hours, which supports once daily dosing, 3) no significant drug – drug interactions which would limit the use of emtricitabine in combination therapy, 4) comparable safety and efficacy to lamivudine, 5) a low incidence of M184V mutations. This is an extremely important observation which suggests that emtricitabine could increase the durability of oxathiolane nucleoside analog-containing drug regimens.

Although the HBV clinical development program is just entering the pivotal phase, there are already data to suggest that at the same 200 mg qd dose selected for HIV development, there is a lower incidence of the rtM204V mutation than has historically been reported for lamivudine [55]. As with HIV, this observation holds out the possibility of more durable therapy for the treatment of chronic HBV infection. It also suggests that emtricitabine will be an extremely important drug for the treatment of patients coinfected with HIV and HBV.

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