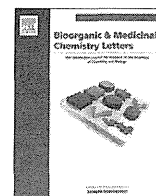


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## 2'-Fluoro-6'-methylene-carbocyclic adenosine phosphoramidate (FMCAP) prodrug: In vitro anti-HBV activity against the lamivudine–entecavir resistant triple mutant and its mechanism of action

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### ABSTRACT

Novel 2'-fluoro-6'-methylene-carbocyclic adenosine (FMCA) monophosphate prodrug (FMCAP) was synthesized and evaluated for its in vitro anti-HBV potency against a lamivudine–entecavir resistant clone (L180M + M204V + S202G). FMCA demonstrated significant antiviral activity against wild-type as well as lamivudine–entecavir resistant triple mutant (L180M + M204V + S202G). The monophosphate prodrug (FMCAP) demonstrated greater than 12-fold (12×) increase in anti-HBV activity without increased cellular toxicity. Mitochondrial and cellular toxicity studies of FMCA indicated that there is no significant toxicity up to 100 μM. Mode of action studies by molecular modeling indicate that the 2'-fluoro moiety by hydrogen bond as well as the Van der Waals interaction of the carbocyclic ring with the phenylalanine moiety of the polymerase promote the positive binding, even in the drug-resistant mutants.

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The chronic HBV infection is strongly associated with liver diseases like chronic hepatic insufficiency, cirrhosis and hepatocellular carcinoma (HCC).<sup>1</sup> According to the World Health Organization (WHO), currently about 2 billion people world-wide have been infected with HBV and more than 350 million live with chronic infection. Acute or chronic outcomes of HBV infection are estimated to cause the deaths of 600,000 people worldwide every year.<sup>2</sup>

Currently, there are several nucleos(t)ide analogues available to treat chronic hepatitis B virus infection.<sup>3–6</sup> The major target of these drugs is to inhibit the viral reverse transcriptase (RT)/DNA polymerase, which is responsible for the synthesis of the minus-strand DNA. Although the currently used agents are well tolerated and effective in suppressing the viral replication for extended periods, the significant rate of virological relapse caused by drug resistance remains a critical issue.

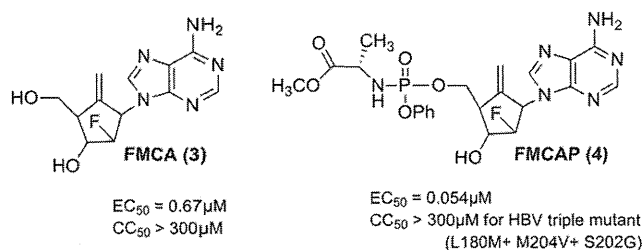
Lamivudine (LVD) was first introduced as the orally active anti-HBV agent in 1998. Lamivudine profoundly suppresses HBV replication in patients with chronic hepatitis B infection; however, lamivudine-resistant HBV (LVDr) was isolated from a significant numbers of patients during the treatment with lamivudine.

Currently, there are several antiviral options exist for these patients viz., to use adefovir or high dose (1.0 mg/day) of entecavir, or more recently tenofovir. However, this resulted in also the development of resistance mutants during the long term therapy. At present, entecavir is the most prescribed drug, and is recommended for patients with the wild-type as well as for those harboring adefovir and lamivudine-resistant strains. However, recent clinical studies by Tanaka and his co-workers suggested that the entecavir mutant in the lamivudine-resistant patients (L180M + M204V + S202G) causes a viral breakthrough: 4.9% of patients at baseline increases to 14.6%, 24% and 44.8% at weeks 48, 96 and 144, respectively.<sup>7</sup> Therefore, it is of great interest to discover novel anti-HBV agent, which is effective against lamivudine- and entecavir-resistant triple mutants (L180M + M204V + S202G).

The potency of a nucleos(t)ide analogue is determined by its ability to serve as a competitive inhibitor of the HBV polymerase relative to that of the natural substrate, the nucleotide triphosphate.<sup>8</sup> However, host cellular kinases limit the pharmacological potency of nucleoside analogues by phosphorylation to their corresponding triphosphates. Particularly, the initial kinase action on the nucleoside to the monophosphate is the rate-limiting step. However, many synthetic nucleosides are not phosphorylated or the rate of phosphorylation is very slow due to the structural requirement of the kinases, resulting in only generating a low quantity of the triphosphate. To overcome this phosphorylation issue, nucleoside phosphoramidate prodrugs have been introduced,<sup>8,9</sup> which

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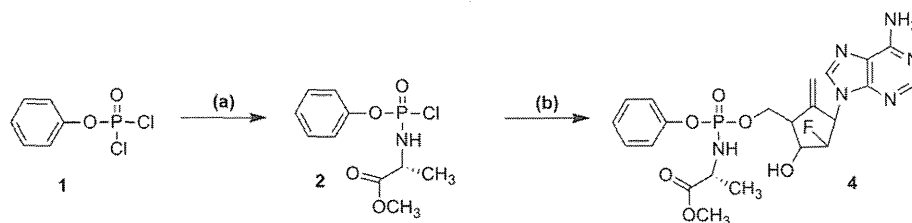
**Figure 1.** Structures of 2'-fluoro-6'-methylene-carbocyclic adenosine (FMCA; **3**) and its prodrug (FMCAP; **4**).

can bypass the rate-limiting first step of monophosphorylation. Phosphoramidate prodrugs have demonstrated to enhance the nucleoside potency in cell culture as well as in patients.<sup>10,11</sup> This methodology greatly increases the lipophilicity of the nucleotide to increase the cell penetration as well as to target the liver cells in vivo.

In this communication, we present that a FMCA phosphoramidate prodrug is such an agent, which can potentially be used for the treatment of patients who experience viral breakthrough due to the triple mutants caused by the use of lamivudine and entecavir.

In our previous report, we have demonstrated that the novel carbocyclic adenosine analog **3** (FMCA Fig. 1) exhibits significant anti-HBV activity against wild type as well as adefovir/lamivudine resistant strains.<sup>12</sup> The present study describes the synthesis and antiviral evaluation of a phosphoramidate of FMCA (FMCAP), which demonstrated the significantly improved in vitro potency. Additionally, we studied its mechanism of action how FMCA-TP can effectively bind to the HBV polymerase by molecular modeling and still exerts the antiviral activity against the lamivudine–entecavir triple mutant (L180M + M204V + S202G).

FMCAP (**4**, Scheme 1)<sup>13</sup> was synthesized using a known method in the literature,<sup>14,15</sup> in which the phosphorylation of phenol with phosphorus oxychloride generates phenyl dichlorophosphate **1**, which was coupled with L-alanine methyl ester in the presence of tri-ethyl amine in dichloromethane to give chlorophosphoramidate reagent **2**, which, in turn, was coupled with FMCA **3** in the presence of 1-methyl imidazole in THF to furnish the phosphoramidate **4** in good yield.

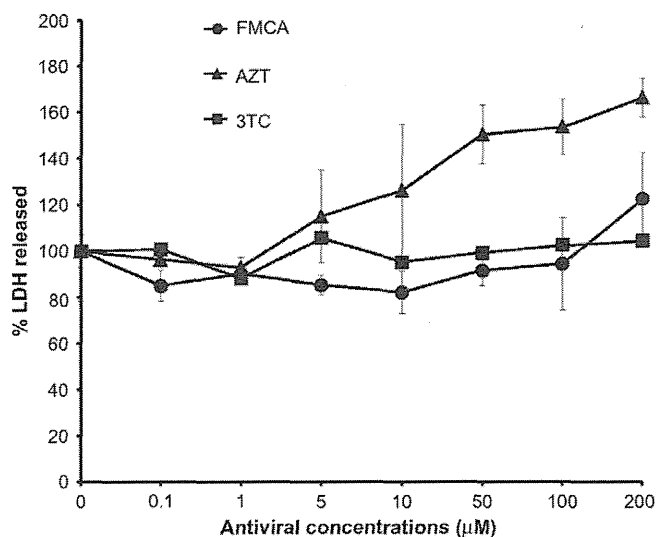


**Scheme 1.** Reagent and conditions: (a) L-alanine methyl ester hydrochloride, Et<sub>3</sub>N, CH<sub>2</sub>Cl<sub>2</sub>; (b) FMCA (**3**), NMI, THF, rt overnight.

**Table 1**

In vitro anti-HBV activity of FMCA **3**, FMCAP **4**, lamivudine and entecavir against wild-type and entecavir drug-resistant mutant (L180M + M204V + S202G) in Huh7 cells

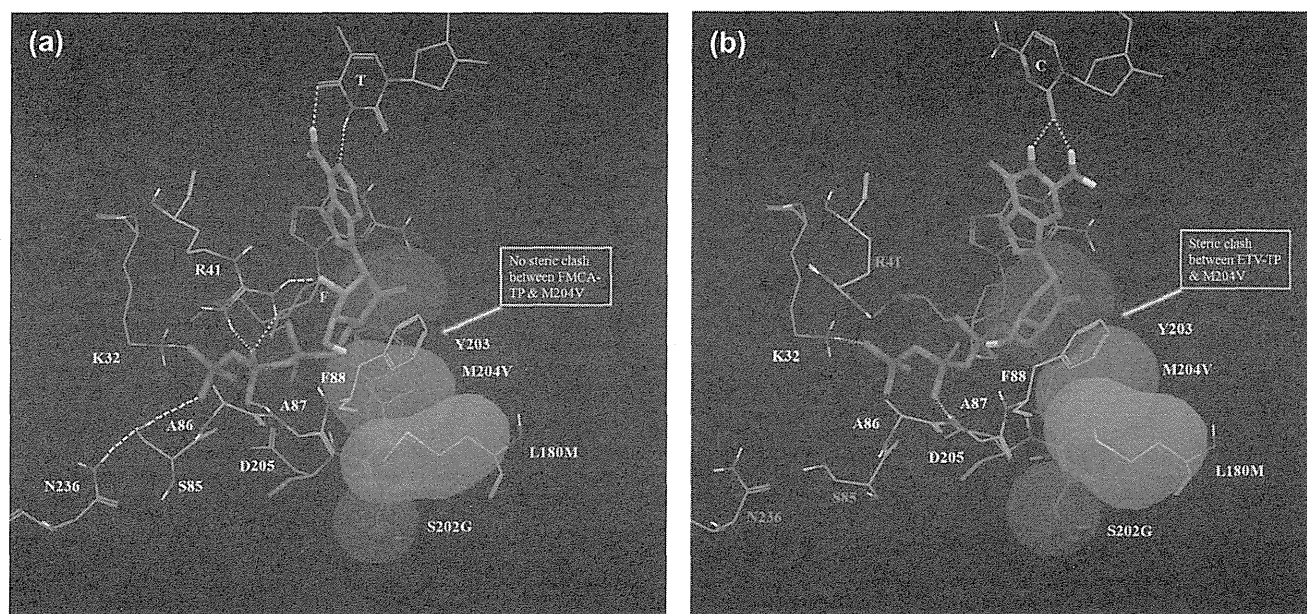
Compounds	HBV Strains			
	EC <sub>50</sub> (μM)	EC <sub>90</sub> (μM)	CC <sub>50</sub> (μM)	L180M + M204V + S202G EC <sub>50</sub> (μM)
FMCA <b>3</b>	0.548 ± 0.056	6.0 ± 0.400	>300	0.67
FMCAP <b>4</b>	0.062 ± 0.011	0.46 ± 0.060	>300	0.054
Lamivudine	0.056 ± 0.003	0.142 ± 0.008	>300	>500 <sup>17</sup>
Entecavir	0.008	0.033	28	1.20 <sup>16</sup>



**Figure 2.** Mitochondrial toxicity of FMCA **3**, AZT and 3TC through lactate dehydrogenase release (LDH) assay.

FMCA **3** and FMCAP **4** were evaluated in vitro against the wild-type as well as the lamivudine–entecavir resistant clone (L180M + S202I + M202V). The FMCA **3** and FMCAP **4** demonstrated significant anti-HBV activity ( $EC_{50}$  0.548 ± 0.056 & 0.062 ± 0.011 μM, respectively) against the wild-type virus, while lamivudine and entecavir also demonstrated potent anti-HBV activity ( $EC_{50}$  0.056 ± 0.003 & 0.008 μM, respectively) (Table 1). It is noteworthy to mention that the anti-HBV potency of FMCAP (**4**) was increased to eight-fold (8×) in comparison to that of FMCA **3**, which indicates the importance of the initial phosphorylation of the nucleoside.

FMCA **3** and FMCAP **4** were further evaluated for their in-vitro antiviral potency against a lamivudine–entecavir resistant clone (L180M + M204V + S202G). It was observed that the anti-HBV potency of both FMCA **3** and FMCAP **4** ( $EC_{50}$  0.67 & 0.054 μM, respectively) were maintained against the resistant clone, and furthermore, the anti-HBV activity of FMCAP **4** was enhanced a 12-fold (12×) with respect to that of FMCA without significant enhancement of cellular toxicity. It was also noteworthy to mention that the anti-HBV potency of entecavir against the mutant



**Figure 3.** (a) FMCA-TP binding mode in ETVr (L180M + M204V + S202G); and (b) ETV-TP binding mode in ETVr (L180M + M204V + S202G) and there is a steric hindrance. Yellow dotted lines are hydrogen bonding interactions ( $<2.5\text{ \AA}$ ). The Van der Waals surface of L180M is colored yellow. The Van der Waals surface of M204V is shown in spring green. The Van der Waals surface of S202G is colored orange. The exocyclic double bond is shown blue color.

**Table 2**

MBAE (multi-ligand bimolecular association with energetics) calculation of FMCA-TP and ETV-TP after Glide XP docking<sup>21</sup> and energy minimization<sup>22</sup>

Strains	Compounds	Energy difference results ( $\Delta E$ , kcal/mol)		
		Total energy	VdW <sup>a</sup>	Electrostatic
Wild-type	FMCA-TP	-588.05	375.78	-6341.08
	ETV-TP	-597.25	350.35	-6009.65
ETVr (L180M + M204V + S202G)	FMCA-TP	-591.54	359.91	-6245.68
	ETV-TP	-320.28	248.82	-4831.12

<sup>a</sup> Van der Waals interaction.

was reduced by 150-fold ( $EC_{50}$  1.2  $\mu\text{M}$ ) in comparison to wild type.<sup>16</sup>

In the preliminary mitochondrial toxicity studies in HepG2 cells by measuring the lactic dehydrogenase release,<sup>18</sup> FMCA **3** did not exhibit any significant toxicity up to 100  $\mu\text{M}$  like lamivudine (3TC), while azidothymidine (AZT) shows the increase of toxicity (Fig. 2).

In our previous report, we described molecular modeling studies for favorable anti-HBV activity of FMCA-TP in wild-type as well as in N236T adefovir resistant (ADVr) mutant.<sup>12</sup> In the current studies, it was of interest to know how the FMCA and its prodrug maintain the anti-HBV activity against ETVr triple mutant (L180M + M204V + S202G) in comparison to entecavir. Therefore, molecular modeling studies were conducted to obtain the insight of the molecular mechanism of FMCA-TP by using the Schrodinger Suite modules.<sup>19</sup> A previously described homology model was used to further explore the impact of the ETVr to the HBV-RT.<sup>12</sup> The homology model of HBV-RT was constructed based on the published X-ray crystal structure of HIV reverse transcriptase (PDB code: 1RTD).<sup>20</sup>

The binding mode of FMCA-TP and ETV-TP in ETVr (L180M + M204V + S202G) HBV-RT are depicted in Figure 3a and b, respectively. Their MBAE (multi-ligand biomolecular association with energetics)<sup>22</sup> calculations of FMCA-TP (total energy, wt -588.05 & ETVr -591.54 kcal/mol) and ETV-TP (total energy, wt -597.25 & ETVr -320.28 kcal/mol) after glide XP (extra precision) docking<sup>21</sup> and energy minimization in ETVr HBV-RT are shown in

Table 2. The triphosphate of FMCA-TP forms all the network of hydrogen bonds with the active site residues (Fig. 3a), K32, R41, S85 & A87 in the similar manner as in wild-type,<sup>12</sup> whereas ETV-TP lose the hydrogen bonding with R41 & S85. The  $\gamma$ -phosphate of FMCA-TP maintains a critical H-bonding with the OH of S85 with connection of hydrogen bonds between S85 and N236 in ETVr HBV-RT also. However,  $\gamma$ -phosphate ETV-TP does not maintain this critical H-bonding with S85 and N236 (Fig. 3b).

The carbocyclic ring with an exocyclic double bond of FMCA-TP and ETV-TP makes the favorable Van der Waals interaction with F88 in ETVr HBV-RT (Fig. 3a and b). There is no steric clash in between exocyclic double bond of FMCA-TP and M204V residue, whereas ETV-TP exocyclic double bond has steric clash with M204V residue in ETVr HBV-RT. The 2'-fluorine substituent in the carbocyclic ring of FMCA-TP appears to promote an additional binding with the NH of R41 guanidino group as shown in Figure 3a, which is in agreement with the antiviral activity of FMCA-TP shown in Table 1. Overall, the modeling studies can qualitatively explain the favorable anti-HBV activity of FMCA-TP against ETVr mutant (L180M + M204V + S202G) in comparison to entecavir as shown in Table 1.

In conclusion, 2'-fluoro-6'-methylene-carbocyclic adenosine phosphoramidate prodrug (FMCA-TP) was synthesized, which demonstrated the significantly increased anti-HBV potency relative to the parent compound, FMCA in vitro. Molecular modeling studies delineated the mechanism of FMCA-TP and how it can effectively bind to the lamivudine-entecavir resistant triple mutant resulting

in maintaining the anti-HBV activity against the mutant. Furthermore, FMCA has been studied for the release of lactic dehydrogenase for potential mitochondrial toxicity and found that no significant increase of toxicity of FMCA compared with other commonly used anti-HIV nucleoside drugs. Very recently, a preliminary in vivo study in chimeric mice harboring the triple mutant, FMCAP was found to reduce HBV viral load while entecavir did not (data not shown). In view of these promising anti-HBV activities and non-toxicity of FMCAP as well as the interesting mechanism of antiviral activity, the chiral synthesis of FMCAP and its mitochondrial toxicity studies for preclinical investigation are warranted.

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13. **Compound 4**:  $^1\text{H}$  NMR (500 Mz,  $\text{CDCl}_3$ )  $\delta$  8.35 (s, 1H), 7.86 (d,  $J = 3.0$  Hz, 1H), 7.34–7.15 (m, 5H), 5.95 (m, 3H), 5.26 (d,  $J = 8.0$  Hz, 1H), 5.01–4.90 (m, 1H), 4.83 (s, 1H), 4.50–4.41 (m, 2H), 4.25–4.04 (m, 3H), 3.71 (s, 3H), 3.07 (s, 1H), 1.40 (d,  $J = 6.5$  Hz, 3 H);  $^{19}\text{F}$  NMR (500 MHz,  $\text{CDCl}_3$ )  $\delta$  -192.86 (m, 1F);  $^{13}\text{C}$  NMR (125 MHz,  $\text{CDCl}_3$ )  $\delta$  171, 159.0, 156.5, 152.5, 150.4, 142.9, 130.1, 121.2, 120.3, 106.7, 102.4, 72.2, 71.1, 62.3, 51.9, 46.3, 43.9, 19.1;  $^{31}\text{P}$  NMR (202 MHz,  $\text{CDCl}_3$ ):  $\delta$  2.67, 2.99. Anal. Calcd For  $\text{C}_{22}\text{H}_{26}\text{FN}_6\text{O}_6\text{P} \cdot 0.5\text{H}_2\text{O}$ : C, 49.91; H, 5.14; N, 15.87; Found C, 49.84; H, 5.06; N, 15.22.
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厚生労働科学研究費補助金 B型肝炎創薬実用化研究事業  
人工キメラ遺伝子と肝臓特異的な輸送担体の開発を基盤とした  
肝臓内 HBVDNA 不活化を目指した新規治療法の開発  
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