Hepatitis B virus (HBV) is a small, partially double-stranded DNA virus and a prototype member of the hepadnavirus family. HBV is a causative agent of both acute and chronic hepatitis. The World Health Organization lists hepatitis B as one of the 10 leading killer diseases and estimates that 350 million people are chronically infected with HBV. Until 1999 the only therapy approved for chronic HBV infection was alpha interferon, and this treatment was useful only for a small minority of Asian patients (13). The hepadnavirus has a unique replication cycle. Following entry into a hepatocyte, the viral genome matures to a single covalently closed circular DNA by an unknown enzyme derived from the host cell and/or hepadnavirus polymerase. The covalently closed circular DNA is translocated into the nucleus and serves as the template for several viral RNAs (4, 33). A hepadnavirus polymerase, which is encoded by a gene in the longest 3.5-kb pregenome RNA, transcribes full-length negative-strand HBV genome from a pregenome RNA within nucleocapsids (3, 30, 35). Sequentially, the polymerase exerts its DNA-dependent DNA polymerase activity to synthesize the positive-strand HBV genome (28). Since the replication cycle of hepadnavirus genome is dependent upon the action of its own polymerase, HBV polymerase could be a good target for an antiviral therapy.

Recently, lamivudine was approved to treat chronic HBV infection (11, 16, 18). Lamivudine is a dideoxycytidine analogue that is active against human immunodeficiency virus (HIV) and HBV (7, 12). It is also shown that lamivudine triphosphate acts as a chain terminator against viral DNA synthesis (5, 37). However, prolonged lamivudine treatment results in the emergence of lamivudine-resistant HBV mutants in 17 to 46% of patients treated for 1 year and more than 50% of patients within 2 years of treatment (1, 11, 16, 20). The emergence of drug-resistant HBV emphasizes the need to develop other antiviral agents and therapeutic strategies. One of the candidates, adefovir dipivoxil, which is an oral prodrug of adefovir (PMEA) and is active against HIV and HBV replication (15, 27, 36), is now in phase III clinical trials to treat HBV infection (31). Although adefovir dipivoxil is suggested to be active against lamivudine-resistant HBV strains (9, 10, 22, 24), nephrotoxicity, characterized by changes in renal function laboratory markers, is observed among HIV patients treated long-term with adefovir dipivoxil (21).

We synthesized more than 100 derivatives of phosphonomethoxyethylpurine and evaluated anti-HBV activity. The absorbability of test compounds was also examined using ex vivo samples from mice and rats given the drug orally (34). Finally, we found 2-amino-6-arylthio-9-phosphonomethoxyethylpurine bis(2,2,2-trifluoroethyl) ester derivatives that showed high antiviral activity and apparent absorption in the animals. In this report, we evaluated the in vitro antiviral properties of MCC-478, 2-amino-6-(4-methoxyphenylthio)-9-[2-(phosphonomethoxy)ethyl]purine bis(2,2,2-trifluoroethyl) ester. Since the bis(2,2,2-trifluoroethyl) ester group was used to improve the oral bioavailability of the phosphonomethoxyethylpurine derivatives and might be hydrolyzed in vivo to give its monoester and free phosphonic acid, the monoester and free phosphonic acid derivatives of MCC-478 were also tested. In order to investigate the toxicological profile of MCC-478, cytotoxicity and reduction of the mitochondrial DNA content was evaluated. It was shown that MCC-478 is a potent inhibitor of HBV replication, and its antiviral profile is specific to HBV.

**Materials and Methods**

**Compounds.** The following test compounds were chemically synthesized at Mitsubishi Pharma Corporation: MCC-478 (2-amino-6-(4-methoxyphenylthio)-9-[2-(phosphonomethoxy)ethyl]purine bis(2,2,2-trifluoroethyl) ester), monoester derivative 1 (M-1) (2-amino-6-(4-methoxyphenylthio)-9-[2-(phosphonomethoxy)ethyl]purine 2,2,2-trifluoroethyl ester), free phosphonic acid derivative 1 (F-1) (2-amino-6-(4-methoxyphenylthio)-9-[2-(phosphonomethoxy)ethyl]purine), and monoester derivative 2 (M-2) (2-amino-6-(4-hydroxyphenylthio)-9-[2-(phos-
MCC-478, a Novel and Specific Inhibitor of HBV

RESULTS

Antiviral activities of MCC-478 and other nucleoside or nucleotide analogues. We first attempted to evaluate anti-HBV activities of MCC-478, lamivudine, and PMEA in HB611 cells, which is a stable transfected cell line producing HBV.

HB611 cells were treated with various concentrations of MCC-478, lamivudine, and PMEA. The antiviral activities of MCC-478 (10 μM) and lamivudine (3 μM) were evaluated against HBV replication in HB611 cells, which is a stable transfected cell line producing HBV.

The results of this study indicate that MCC-478 is a novel and specific inhibitor of HBV, showing high antiviral activity against HBV replication in vitro.
478, lamivudine, or PMEA (both lamivudine and PMEA are used as reference drugs in many studies for anti-HBV agents). The cells in five wells on a 24-well plate were treated independently at each concentration for 8 days and harvested on the last day of culture. After total cellular DNA was prepared, HBV DNAs derived from both replication intermediates and integrated HBV genome were detected and analyzed by Southern blotting. Percent control values were calculated from the untreated controls (U). The bands derived from integrated HBV are indicated by the arrows. The mean value at each concentration is plotted on the graph, and the standard deviations are indicated by the error bars.

Figure 2 shows the average percent control values of HBV DNA replication in cells treated with drugs at each concentration. Also, WinNonlin version 1.1 (Scientific Consulting Inc.) was used to calculate the 50% and 90% effective concentrations (EC50 and EC90, respectively) of MCC-478 and lamivudine. The EC50 and EC90 of MCC-478 were 0.027 and 0.24 μM, respectively, while those of lamivudine were 2.2 and 7.8 μM, respectively. The EC50 of PMEA could not be calculated, since PMEA gave percent control values below 50 even at the lowest concentration (0.1 μM). These results clearly suggested that MCC-478 was a potent inhibitor of HBV DNA replication.

Comparison of the anti-HBV activities of the hydrolyzed derivatives of MCC-478. Anti-HBV activity was also observed in sera from mice given MCC-478 orally (data not shown). Since some of the bisalkyl ester prodrugs of PMEA were not cleaved completely to a free phosphonic acid form (29), two monoester derivatives (M-1 and M-2) and one phosphonic acid derivative (F-1) of MCC-478 (Fig. 1) were putative metabolites in serum. In order to understand their contribution to efficacy in vivo, they were synthesized and tested in HB611 cells. As shown in Fig. 3, the anti-HBV activities of M-1 and F-1 were similar to that of MCC-478, and the anti-HBV activity of M-2 was similar to that of M-1.

Cytotoxicity and anti-HIV activity of MCC-478. The 50% cytotoxic concentration (CC50) of each test compound was determined in the HuH-6 cell line, the parent cell line of

FIG. 2. Inhibitory effects of MCC-478, PMEA, and lamivudine against HBV replication in HB611 cells. Cells were treated with the indicated concentrations of test compound for 8 days and harvested on the last day of culture. After total cellular DNA was prepared, HBV DNAs derived from both replication intermediates and integrated HBV genome were detected and analyzed by Southern blotting. Percent control values were calculated from the untreated controls (U). The bands derived from integrated HBV are indicated by the arrows. The mean value at each concentration is plotted on the graph, and the standard deviations are indicated by the error bars.

FIG. 3. Comparison of the inhibitory effects of MCC-478, M-1, M-2, and F-1 against HBV replication in HB611 cells. Cells were treated with the indicated concentrations of test compound for 8 days. Anti-HBV activity was evaluated as indicated in the legend to Fig. 2. The mean value at each concentration is plotted on the graph, and the standard deviations are indicated by the error bars.
HB611. MCC-478 and its derivatives were well tolerated (Table 1). To evaluate the specificity for antiviral activity, anti-HIV activities of MCC-478, PMEA, and lamivudine were determined by examining the inhibition of the HIV-induced cytopathic effect in the MT-4 cell line. Treatment with MCC-478 showed no significant selectivity between the cytotoxicity against mock-infected MT-4 cells and the inhibition of HIV-induced cytopathic effect, e.g., 16 and 11%, respectively, even at the highest concentration. The EC₅₀s and CC₅₀s of the test compounds in MT-4 cells are summarized in Table 1. These results suggested that anti-HIV activity of MCC-478 was much weaker than anti-HBV activity, while PMEA retained anti-HBV and anti-HIV activity.

**Effect of MCC-478 on mitochondrial DNA content.** Therapies with nucleoside analogues sometimes cause mitochondrial dysfunction, which could be derived from incorporation of the nucleoside analogue into mitochondrial DNA (8) or reduction in mitochondrial DNA content (6). In this study, the effects of MCC-478 and its hydrolyzed derivatives on mitochondrial DNA content were examined, using 2',3'-dideoxycytidine (ddC) as a positive control. HepG2 cells were treated with each test compound at a final concentration of 0.1, 1.0, or 10 μM for 8 days. Mitochondrial DNA content was examined by dot blot hybridization. Treatment with MCC-478, M-1, M-2, or PMEA showed no reduction in mitochondrial DNA content, even though treatment with ddC at every concentration reduced mitochondrial DNA content to nearly half that of the untreated control (Fig. 4).

**DISCUSSION**

MCC-478 is a novel phosphonomethoxyethylpurine derivative which has an arylthio group at position 6 of the purine base. Also, MCC-478 has 2-aminopurine nucleobase structure, which indicates that it is more closely related to 9-[2-(phosphonomethoxy)ethyl]guanine (PMEG) than to PMEA. However, introduction of the arylthio group at position 6 of the 2-aminopurine base has been demonstrated to endow the purine base with a higher specificity against HBV and low cytotoxicity comparable to that of PMEA (Table 1), while PMEG

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**TABLE 1. Summary of the antiviral and cytotoxic effects of the test compounds**

<table>
<thead>
<tr>
<th>Test compound</th>
<th>HBV in HB611 cells</th>
<th>HIV-1 in MT-4 cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EC₅₀ (μM)</td>
<td>EC₅₀ (μM)</td>
</tr>
<tr>
<td></td>
<td>Expt 1</td>
<td>Expt 2</td>
</tr>
<tr>
<td>MCC-478</td>
<td>0.027</td>
<td>0.026</td>
</tr>
<tr>
<td>M-1</td>
<td>0.072</td>
<td>0.15</td>
</tr>
<tr>
<td>M-2</td>
<td>0.15</td>
<td>0.29</td>
</tr>
<tr>
<td>PMEA</td>
<td>&lt;0.1</td>
<td></td>
</tr>
<tr>
<td>Lamivudine</td>
<td>2.2</td>
<td></td>
</tr>
</tbody>
</table>

* a EC₅₀ and EC₉₀ of anti-HBV activity were calculated from the result of the experiments shown in Fig. 2 and 3.
* b Concentrations were calculated on the basis of the molecular weight of each compound.
* c NT, not tested.
is known to have higher cytotoxicity than PMEA (17). These results suggest that substitution at position 6 of the purine base may alter the biological and toxicological profiles of the phosphonomethoxyethyl purine derivatives.

It was reported that adefovir dipivoxil is primarily hydrolyzed to the monoester form and is further metabolized to adefovir (PMEA) in the presence of either cells or serum (27). This finding may mean that there was an esterase to hydrolyze both bis(pivaloyloxymethyl) and mono(pivaloyloxymethyl) ester forms in both serum and cells. In the case of MCC-478, which is a bis(2,2,2-trifluoroethyl) ester, it was hydrolyzed to only the monoester form, even in the presence of serum from humans or rats (data not shown). In addition, major metabolites in rats and monkeys were found to be the monoester forms of MCC-478 (M-1 and M-2), rather than the free phosphonic acid form (F-1) (Y. Yamaguchi, personal communication). On the other hand, the free phosphonic acid form, PMEA, was identified as the major metabolite in sera from the animals given adefovir dipivoxil orally. This speculated difference of metabolites between MCC-478 and adefovir dipivoxil in vivo suggested the necessity of examining the antiviral activities of these metabolites in order to estimate their efficacy in vivo. Here, not only the monoester forms but also the free phosphonic acid form were recognized to have antiviral activities similar to that of MCC-478 in HB611 cells (Fig. 3). The anti-HBV activities of these MCC-478 derivatives were high enough to suggest that they could contribute to in vivo efficacy of MCC-478. These results also indicated that the mono(2,2,2-trifluoroethyl) ester forms of MCC-478 may be hydrolyzed only within the cell and further metabolized to an active form of MCC-478.

In addition to the antiviral profile of MCC-478, it was also shown that the cytotoxic effects of MCC-478 and its hydrolyzed derivatives were quite low (Table 1). It may also be important to evaluate mitochondrial toxicity to understand the biological profile of a nucleotide analogue, since long-term treatment with antiviral nucleoside analogues could give rise to delayed and possibly severe mitochondrial toxicity (19). Nucleoside and nucleotide analogs can be classified into two types on the basis of their mechanism of mitochondrial toxicity. The first type of nucleoside analogue, such as fialuridine, has a 3'-hydroxyl group (3'-OH), and mitochondrial toxicity is associated with nucleoside incorporation into the mitochondrial DNA (8). The second type of nucleoside analogue, such as ddC, does not have a 3'-OH group, and mitochondrial toxicity is associated with reduced mitochondrial DNA content by chain termination (6). Since MCC-478 and its hydrolyzed derivatives do not have 3'-OH, they may belong to the latter type of nucleoside analogues. However, as shown in Fig. 4, there was no evidence of inhibition of mitochondrial DNA replication. This lack of evidence of inhibition may mean that MCC-478 and its metabolites did not have inhibitory activity against DNA polymerase γ. A chain-terminating analysis using DNA polymerase α also revealed that neither F-1 nor its diphosphate showed chain-terminating activity against DNA polymerase α; however, PMEA diphosphate was thought to have chain-terminating activity at the same concentrations (data not shown). These results emphasize the specific antiviral profiles of MCC-478 and its derivatives. Interestingly, the diphosphates derived from MCC-478 were not found intracellularly, so far as we tried. While there is a possibility that the diphosphates might be a very potent and selective inhibitor of hepadnavirus polymerase, MCC-478 and its derivatives might not require phosphorylation for biological activity. More studies of intracellular metabolism and active metabolites would be needed to elucidate the mechanisms of MCC-478 antiviral activity.

Lamivudine is the first approved nucleoside analogue for treatment of HBV infection. It is known to have potent activity against both HBV and HIV replication (16). However, the rapid emergence of viruses resistant to lamivudine in HIV-infected patients is also known, even with short-term therapy, and recently a similar phenomenon was observed with lamivudine monotherapy (1, 11, 20). Most lamivudine-resistant virus strains have methionine (M) substituted for isoleucine (I) or valine (V) on the YMDD (tyrosine-methionine-aspartate-aspartate) motif in catalytic domains of HIV polymerase (14) and hepadnavirus polymerase (1). Another drug-resistant substitution of leucine (L) to M in B domain (L528M) is reported from a clinical trial of famciclovir in HBV-infected patients (25). The L528M mutation frequently accompanies the YMDD mutation, and HBV carrying these two mutations is cross-resistant to lamivudine and famciclovir (10). In the case of HIV, a steric hindrance between the mutant amino acid side chain and lamivudine triphosphate is suggested from the molecular model analysis of comparisons of crystal structures of the YMDD mutant and wild-type HIV polymerases with or without double-strand DNA (26). Moreover, this molecular model suggested that a steric hindrance between the mutant amino acid side chain and the lamivudine sugar ring might be expected in nucleoside analogues with β-1,4-ring configurations. In the case of HBV, a recent study reporting molecular modeling analysis using the HBV polymerase homology model (9) also supports this concept. Ono et al. (22) evaluated the inhibitory effects of a panel of 11 nucleoside and nucleotide analogues on HBVs with one or two mutations by in vitro full-length HBV DNA transfection and found that only a few nucleoside analogues, including PMEA, are active against lamivudine-resistant HBVs. Since MCC-478 was shown to be a novel nucleoside analogue having potent and selective anti-HBV activity, even if it was a derivative of phosphonomethoxyethylpurine, like PMEA, MCC-478 should be further examined for an inhibitory profile against drug-resistant HBVs, including lamivudine-resistant HBVs.

The aim of this work was to evaluate MCC-478 and its derivatives as potent and specific agents for the treatment of HBV infections. Although many studies have to be done to understand the mechanism of action, toxicological pharmacokinetic profiles, and so on, MCC-478 could be a promising new anti-HBV agent.

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