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***In vitro* characterisation of the anti-hepatitis B virus (HBV) activity and cross-resistance profile of 2', 3'-dideoxy-3'-fluoroguanosine.**

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ABSTRACT

The fluorinated guanosine analog 2', 3'-dideoxy-3'-fluoroguanosine (FLG) was shown to inhibit wild-type (WT) human hepatitis B virus (HBV) replication in a human hepatoma cell line HepG2.2.15 expressing permanently HBV. Experiments performed in the duck model of HBV infection also showed its *in vivo* antiviral activity. The aim of this study was to determine the mechanism of inhibition of FLG on HBV replication and its profile of antiviral activity against different HBV or DHBV drug-resistant mutants. In a cell free system assay allowing the expression of an enzymatically active duck hepatitis B virus (DHBV) reverse transcriptase, FLG-triphosphate (FLG-TP) was a more potent inhibitor of WT DHBV viral minus strand DNA synthesis compared to lamivudine and showed a similar activity compared to adefovir. FLG-TP was most likely a competitive inhibitor of dGTP incorporation and a DNA chain terminator. In Huh7 cells transiently transfected with different HBV constructs, FLG inhibited similarly the intracellular replication of WT, lamivudine-resistant, adefovir-resistant and lamivudine+adefovir-resistant HBV mutants. These results were consistent with those obtained in the DHBV polymerase assay using the same drug-resistant polymerase mutants.

In conclusion, our data provide new insights in the mechanism of action of FLG-TP on HBV replication and demonstrate its inhibitory activity on drug-resistant mutant reverse transcriptases *in vitro*. Furthermore, our results provide the rationale for further clinical evaluation of FLG in the treatment of drug-resistant virus infection, and in the setting of combination therapy to prevent or delay drug resistance.

INTRODUCTION

The development of nucleos(t)ide analogs that inhibit the hepatitis B virus (HBV) reverse transcriptase (RT) activity has provided new hope in the treatment of chronic hepatitis B. Lamivudine ([-]- β -L-2',3'-dideoxy-3' thiacytidine; also named 3TC), adefovir (bis-pivaloyloxymethyl-9-(2-phosphonyl-methoxyethyl) adenine; also named PMEAs) and entecavir (2-amino-1,9-dihydro-9-[(1*S*, 3*R*, 4*S*)-4-hydroxy-3-(hydroxymethyl)-2-methylenecyclopentyl]-6*H*-purin-6-one, monohydrate; also named ETV) are currently the three nucleos(t)ide analogs approved for the treatment of HBV infection. However, the slow kinetics of viral clearance during 3TC therapy and the spontaneous viral genome variability lead to the selection of 3TC-resistant mutants. Up to 66 % of the patients develop viral resistance after 4 years of 3TC therapy [13]. The M204V/I mutations frequently associated with 3TC breakthrough are located in the tyrosine-methionine-aspartate-aspartate (YMDD) motif within the C domain of the RT and confer 3TC-resistance [1]. They are often associated with compensatory mutations (L180M or V173L) in the B domain of the RT [18, 5]. ETV has been very recently approved in the USA. It remains efficient against the 3TC-resistant mutants *in vitro* and *in vivo* [16, 18, 26]. However, in clinical trials for patients infected with 3TC-resistant HBV strains, prolonged ETV treatment has led to the selection of complex mutants harboring specific polymerase mutations in addition to 3TC-resistant ones associated with a decreased ETV susceptibility [26]. 3TC-resistant mutants remain sensitive to PMEAs *in vitro* [23, 30] and *in vivo* [19]. However, recent reports showed that, after two years of therapy, a novel N236T mutation located in the D domain of the HBV polymerase could be selected although at a lower rate than the 3TC-resistant mutants. This mutation confers a 4 to 23-fold reduced susceptibility to PMEAs but remains sensitive to 3TC [2, 27].

Other nucleoside analogs are currently in phase III clinical trials, i.e. emtricitabine ((-)- β -2', 3'-dideoxy-5-fluoro-3'-thiacytidine; also named -FTC), a close analog of 3TC, and

telbivudine (1-(2-deoxy- β -L-*erythro*-pentofuranosyl)-5-methylpyrimidine-2,4(1*H*, 3*H*)-dione; also named LdT) [8, 12]. However, 3TC-resistance mutations confer cross-resistance to - FTC and LdT [31]. The development of new HBV inhibitors to rescue drug resistance, or to design new combination strategies aiming at delaying or preventing drug resistance, is thus mandatory. Several anti-HBV compounds are currently under evaluation. One of these, 2', 3'-dideoxy-3'-fluoroguanosine (FLG), is a synthetic deoxyguanosine analog originally developed to inhibit human immunodeficiency virus (HIV) replication [10]. The functional similarity of the HBV and HIV DNA polymerases has led to the examination of FLG as an inhibitor of HBV and duck HBV (DHBV) polymerase activity. The antiviral activity of FLG was investigated *in vitro* and *in vivo* on both HBV and DHBV replication [9, 17, 20]. *In vitro*, FLG was shown to be an efficient inhibitor of both DHBV and HBV replication [20]. *In vivo*, FLG showed a favorable safety profile and inhibited DHBV replication in DHBV infected ducks with a significant reduction of serum DHBV DNA levels [17].

Therefore the aim of our study was to determine the mechanism of action of FLG on HBV replication and its anti-HBV activity profile against lamivudine- and adefovir-resistant mutants that are commonly observed in clinical practice. We used an *in vitro* assay that allows the expression of the DHBV RT to characterize the mechanism of action of FLG-triphosphate (FLG-TP) [32, 33]. The inhibitory effect of FLG-TP was also assessed on DHBV RT harboring mutations conferring resistance to 3TC, PMEA, or to both 3TC and PMEA. Furthermore, the inhibitory effect of FLG was assessed in transiently transfected Huh7 cells on the replication of wild-type (WT) and drug-resistant mutant HBV laboratory strains.

MATERIALS AND METHODS

Antiviral Drugs. FLG and its triphosphate form FLG-TP, 3TC and 3TC-TP, PMEA and PMEA-diphosphate (PMEA-DP) were provided by Medivir (Lunastigen 7, S-141 44 Huddinge, Sweden).

Expression vectors for the study of wild-type and drug-resistant mutants of DHBV and HBV. Wild-type (WT) and mutant HBV and DHBV polymerases were used to study their sensitivity to nucleos(t)ide analogs. Three mutants were studied: a 3TC-resistant mutant (L180M+M204V for HBV corresponding to L488M+M512V for DHBV), a PMEA-resistant mutant (N236T for HBV corresponding to N544T for DHBV) and a mutant harboring both 3TC- and PMEA-resistant mutations (L180M+M204V+N236T for HBV corresponding to L488M+M512V+N544T for DHBV). The WT and the L488M+M512V mutant polymerase genes of DHBV were previously cloned into pHP plasmids under the control of a SP6 promoter to study their enzymatic activity in a cell free system [22]. The N544T mutation was introduced by site directed mutagenesis with the Quickchange kit (Stratagene, La Jolla, CA) into these plasmids to obtain the N544T and the L488M+M512V+N544T DHBV polymerases. Mutated polymerase gene sequences were checked by sequencing. The plasmids pCMV-HBV containing WT or L180M+M204V mutant HBV 1.1 genome length (genotype D, subtype ayw) [3, 22] were modified by site directed mutagenesis with the Quickchange kit to introduce the N236T mutation. The mutated plasmids were then sequenced and EcoRI-NcoI restriction fragments encompassing the L180M+M204V, N236T or L180M+M204V+N236T mutations were transferred into the plasmid pTriEXMod-HBV. This latter construct, that contains 1.1 unit of HBV genome, enables after cell transfection the production of pregenomic RNA under the control of the chicken beta actin promoter and therefore triggers HBV DNA synthesis [6].

An *in vitro* assay for the expression of enzymatically active DHBV reverse transcriptase and evaluation of the inhibitory effect of nucleoside triphosphate analogs.

The enzymatically active DHBV polymerase polypeptide was expressed from plasmids pHP. These constructs contain the WT or mutant (L488M+M512V, N544T or L488M+M512V+N544T) DHBV polymerase gene under the control of Sp6 promoter and the sequence coding for the RNA template of reverse transcription as previously described [4, 28]. The DHBV polymerase gene from each construct was transcribed and translated in a coupled transcription-translation rabbit reticulocyte lysate system (TNT SP6 Coupled Reticulocyte Lysate System, Promega, Charbonnières, France), according to manufacturer's instructions.

The reverse transcription assay was performed as previously described [4, 32, 33]. After translation, the viral polymerase was incubated for 30 min at 30°C with an equal volume of a reaction mix containing 100 mM Tris-HCl pH 7.5, 30 mM NaCl, 20 mM MgCl₂ and a nucleotide mix of which the composition depends on the type of reaction.

The priming reaction corresponds to the synthesis of a short DNA primer, GTAA, for reverse transcription. To study this reaction, the composition of dNTP mix depended on the nucleos(t)ide analog tested. For FLG-TP, only [α^{32} P]-dGTP (0.165 μ mol/L; 3,000 Ci/mm) was used to determine if the drug was able to compete with the incorporation of the first nucleotide (dGTP) in viral minus strand DNA. For the evaluation of PMEADP, the dNTP mix included cold dGTP and dTTP (100 μ mol/L each) with [α^{32} P]-dATP (0.165 μ mol/L; 3,000 Ci/mm).

To study the elongation of viral minus strand DNA, the reaction mixture included the radioactive nucleotide [α^{32} P]-dNTP (0.165 μ mol/L; 3,000 Ci/mm) of which the drug tested is the analog, whereas the 3 other cold dNTPs were used at 100 μ mol/L each. Radiolabelled viral DNA covalently attached to polymerase was analyzed through 0.1% sodium dodecyl

sulfate (SDS)-10% polyacrylamide gels as previously published [4, 33] and the dried gels were exposed to X-ray film. A quantitative dot assay was performed after spotting 2 μ l of the radiolabelled reverse transcription assay mixture onto DE-81 filters (Whatman) as previously described [32]. In each assay, background incorporation was calculated with a control experiment where the reaction was performed without addition of pHP plasmid. All results were expressed in percentage of inhibition of the polymerase activity as compared to the reaction where the polymerase was incubated without any inhibitor. Quantification of densitometric scanning of autoradiograms and experiments with DE-81 filters showed similar results.

To determine whether FLG-TP is a competitive inhibitor of the incorporation of the natural nucleotide [α^{32} P]-dGTP incorporation into nascent viral DNA, the reverse transcription assay described above was performed with [α^{32} P]-dGTP at a final concentration of 0.165 μ M or 0.825 μ M together with increasing concentrations of FLG-TP ranging from 0 to 100 μ M.

To determine whether FLG-TP could inhibit the incorporation of the next nucleotide in nascent viral minus strand DNA by chain termination, we used the WT polymerase expressed in a cell free system with a WT epsilon sequence that allows the synthesis of the short DNA primer GTAA. The dNTP mix was composed of dGTP and [α^{32} P]-TTP (0,165 μ mol/L each). The incorporation of radiolabelled TTP in viral minus strand DNA by the RT in absence or presence of increasing concentrations (0 to 100 μ M) of FLG-TP was analyzed and quantified as already described [4, 33]. Results were compared to the effect of the corresponding dideoxynucleotide (ddGTP) that terminates viral minus strand DNA synthesis after its incorporation into the viral DNA chain.

Analysis of the inhibitory activity of FLG on the replication of wild-type and drug-resistant mutants of HBV in Huh7 cells.

The analysis of FLG antiviral activity was evaluated in comparison with that of 3TC and PMEA after transient transfection of Huh7 cells by either WT, 3TC-resistant (L180M+M204V), PMEA-resistant (N236T) or 3TC- and PMEA-resistant (L180M+M204V+N236T) mutant polymerases. Twelve-well plates cultured cells at a density of 7.10^4 cells/cm² were transfected 1-day post-plating with 0.5 µg/well of pTriEXMod-HBV plasmid containing the WT or a mutant HBV genome, using Fugene 6 transfection reagent (Roche Diagnostics, Mannheim, Germany) according to manufacturer's instruction. Sixty hours post-transfection, treatment with increasing concentrations of drugs was started. Medium was changed daily and drug administration was performed from day 3 to day 8 post-transfection. Cells were then lysed for analysis of intracellular viral DNA. HBV DNA was purified from intracellular core particles as described earlier [11], separated on agarose gels, analyzed by Southern Blot hybridization and quantified after phosphor imager scanning [22].

RESULTS

FLG-TP inhibits the wild-type DHBV reverse transcriptase activity.

Using an *in vitro* assay that allows the expression of an enzymatically active DHBV RT, we analyzed the inhibitory effects of the triphosphate form of FLG (FLG-TP) on viral minus strand DNA synthesis by WT DHBV polymerase in comparison with 3TC-TP and PMEADiphosphate (DP). The incorporation of [$\alpha^{32}\text{P}$]dGTP in presence of increasing concentrations of FLG-TP was reproducibly inhibited in a dose dependent manner (Figure 1A). Moreover, FLG-TP inhibited more efficiently the RT activity of the WT viral polymerase than 3TC-TP ($\text{IC}_{50} = 4.9 \pm 0.5 \mu\text{M}$ for FLG-TP against $8.0 \pm 1.2 \mu\text{M}$ for 3TC-TP) and had a similar inhibitory activity as PMEADP ($\text{IC}_{50} = 4.6 \pm 0.8 \mu\text{M}$), in our experimental conditions (Table 1).

The inhibitory activity of 3TC-TP, PMEADP and FLG-TP was also tested on the synthesis of short DNA primer. During the priming of reverse transcription, the DHBV polymerase synthesizes a short 4-base DNA oligomer by copying an RNA motif located in the bulge of the epsilon stem-loop [14, 25]. The sequence of the DNA primer is GTAA for DHBV. Our results showed that PMEADP was a potent inhibitor of the DNA primer synthesis ($\text{IC}_{50} = 4.9 \mu\text{M} \pm 0.4$), whereas FLG-TP inhibited the priming reaction by only 40 % at 100 μM ($\text{IC}_{50} > 100 \mu\text{M}$) (Figure 1B, Table 1). 3TC-TP was not tested in this priming reaction because the short primer for reverse transcription (GTAA) does not include deoxycytidine.

To determine whether FLG-TP may be a competitive inhibitor of dGTP incorporation in DHBV minus-strand DNA, *in vitro* assay allowing the expression of an enzymatically active DHBV RT was used with radiolabelled [$\alpha\text{-}^{32}\text{P}$]dGTP at a final concentration of 0.165 μM or 0.825 μM . When the concentration of [$\alpha\text{-}^{32}\text{P}$]dGTP was increased by 5-fold, the IC_{50}

of FLG-TP shifted from 4.9 to 12.7 μM (2.6-fold increase), suggesting a competitive inhibitory effect of the drug on dGTP incorporation in viral minus strand DNA (Figure 2). We then asked whether FLG-TP may act as a terminator of viral minus strand DNA chain elongation. The viral polymerase was incubated in presence of 0.165 μM of dGTP and [α - ^{32}P]TTP with increasing concentrations (0 to 100 μM) of FLG-TP or of dideoxyguanosine-triphosphate (ddGTP). Increasing concentrations of FLG-TP and ddGTP both inhibited the incorporation of the next radiolabelled TTP, although ddGTP was a more potent inhibitor than FLG-TP ($\text{IC}_{50} = 0.1 \mu\text{M}$ for ddGTP versus 3.0 μM for FLG-TP) (Figure 3). Altogether these results suggest that FLG-TP is likely to be a competitive inhibitor of the incorporation of the natural substrate, i.e. dGTP, and then inhibits the incorporation of the next nucleotide. However, as shown in Figure 3, [α - ^{32}P]-TTP incorporation was not completely inhibited by neither ddGTP nor FLG-TP. Indeed, at a maximal drug concentration of 100 μM , 13% to 25% of [α - ^{32}P]-TTP were still incorporated in the priming reaction. Moreover, in absence of cold dNTPs, a low level of incorporation of [α - ^{32}P]-TTP was still detectable (data not shown). This suggests that a pool of endogenous dNTPs may allow the priming of reverse transcription in the reticulocyte lysate, or that this reaction may start directly by using TTP instead of dGTP. To test this hypothesis, we attempted to purify the rabbit reticulocyte lysate by exclusion chromatography to eliminate dNTPs before or after the coupled transcription/translation reaction. Despite the depletion of endogenous dNTP, the incorporation of [α - ^{32}P]-TTP was not totally inhibited (data not shown). This suggests either that the depletion of endogenous dNTPs was not complete, or that a minority of the DHBV polymerase polypeptides may prime reverse transcription directly with the TTP in our *in vitro* conditions.

FLG inhibits viral DNA synthesis in Huh7 cells transiently transfected with wild-type HBV genome.

We next assessed the inhibitory activity of FLG on intracellular HBV DNA synthesis *in vitro* by comparison with those of 3TC and PMEA. Huh7 cells were transiently transfected with pTriEXMod-HBV plasmid containing WT HBV genome and treated for 5 days with increasing concentration of FLG, 3TC or PMEA. Intracellular HBV DNA replicative intermediates were then extracted, submitted to Southern Blot analysis, and quantified by phosphorimager scanning densitometry to determine the IC₅₀s of each drug. Results showed that all three nucleos(t)ide analogs inhibited WT HBV replication in a dose dependent manner. According to three independent experiments, the most potent inhibition of intracellular HBV DNA synthesis was observed with 3TC (IC₅₀ = 0.5 ± 0.4 μM), followed by FLG (IC₅₀ = 9 ± 2.5 μM) and PMEA (IC₅₀ = 15 ± 3.4 μM) (Table 2).

The effect of FLG on the cellular viability was evaluated in HuH7 cells by the neutral red assay in comparison with 3TC and PMEA. No significant cytotoxicity was observed with either 3TC, PMEA or FLG at the highest drug concentration used (data not shown).

3TC-resistant, PMEA-resistant and 3TC+PMEA-resistant mutants of DHBV and HBV remain sensitive to FLG.

The enzymatic activity of three DHBV mutant polymerases corresponding to the 3TC-resistant (3TC-R; L488M+M512V), PMEA-resistant (PMEA-R; N544T), and 3TC+PMEA-resistant (3TC+PMEA-R; L488M+M512V+N544T) DHBV mutants was analyzed in presence of increasing concentration of FLG-TP, 3TC-TP and PMEA-DP in comparison to WT DHBV polymerase. 3TC-TP was not active on either the 3TC-R or the 3TC+PMEA-R DHBV polymerases (IC₅₀ > 100μM) but had a similar inhibitory activity on

both WT ($IC_{50} = 8 \pm 1.2 \mu M$) and PME-A-R ($IC_{50} = 11 \pm 1.4 \mu M$) DHBV polymerases (Table 1). PME-A-DP inhibited both WT ($IC_{50} = 4.6 \pm 0.8 \mu M$) and 3TC-R ($IC_{50} = 4.4 \pm 0.9 \mu M$) DHBV polymerases with the same potency, whereas it showed a decreased inhibitory activity against the PME-A-R (IC_{50} of $39 \pm 5.5 \mu M$) or the 3TC+PME-A-R ($IC_{50} = 52 \pm 7.1 \mu M$) DHBV polymerases (Table 1). Interestingly, FLG-TP inhibited similarly the enzymatic activity of WT ($IC_{50} = 4.9 \pm 0.5 \mu M$), 3TC-R ($IC_{50} = 4.8 \pm 0.8 \mu M$), PME-A-R ($IC_{50} = 6.7 \pm 1.3 \mu M$) or 3TC+PME-A-R ($IC_{50} = 7.6 \pm 1.9 \mu M$) mutant DHBV polymerases (Figure 1.A, Table 1).

The inhibitory activity of FLG was also evaluated on the replication of 3TC-resistant (3TC-R; L180M+M204V), PME-A-resistant (PME-A-R; N236T) or 3TC+PME-A-resistant (3TC+PME-A-R; L180M+M204V+N236T) HBV mutants in comparison to the WT HBV in transiently transfected Huh7 cells. The same experiments were performed in parallel with 3TC or PME-A administration as a control. 3TC inhibited similarly the viral genome replication of both WT and PME-A-R HBV strains (IC_{50} s = $0.5 \pm 0.4 \mu M$ and $0.8 \pm 0.9 \mu M$, respectively) whereas 3TC-R and 3TC+PME-A-R HBV mutants were resistant to 3TC ($IC_{50} > 100 \mu M$) (Table 2). PME-A inhibited both WT and 3TC-R HBV strains with comparable IC_{50} s ($IC_{50} = 15 \pm 3.4 \mu M$ and $12 \pm 4.5 \mu M$, respectively). However, the PME-A-R mutant had a 2.3 fold decreased susceptibility to PME-A ($IC_{50} = 35 \pm 5.5 \mu M$) compared to WT HBV. The 3TC+PME-A-R mutant was resistant to both 3TC and PME-A ($IC_{50} > 100 \mu M$). Interestingly, all three drug-resistant HBV mutants remained sensitive to FLG with similar IC_{50} s ($IC_{50} = 8 \pm 3.8 \mu M$ for 3TC-R, $13 \pm 3.4 \mu M$ for PME-A-R and $15 \pm 6.8 \mu M$ for 3TC+PME-A-R mutant) as compared to WT HBV ($9 \pm 2.5 \mu M$) (Figure 4, Table 2).

DISCUSSION

In this study, we investigated the mechanism of action and the antiviral activity profile of FLG on the replication of WT and drug-resistant mutants of DHBV and HBV polymerases *in vitro*.

With respect to the mechanism of inhibition of viral DNA synthesis by FLG, we gained new insight by assessing the effect of FLG-TP on the RT activity of an enzymatically active WT DHBV polymerase expressed in a cell free system. First, the study of the priming of the reverse transcription revealed that FLG-TP was a weak inhibitor of this reaction compared to PMEADP (Table 1). This contrasts with other guanosine analogs such as 2' carboxydeoxyguanosine and entecavir that were shown to inhibit the priming of hepadnavirus reverse transcription [4, 21]. However, FLG inhibited significantly the elongation of viral minus strand DNA in a dose dependent manner. Compared to other nucleoside triphosphate analogs, FLG-TP was as active as PMEADP and 1.7-fold more potent than 3TC-TP to inhibit this reaction. Altogether, these results suggest that FLG-TP might reach the catalytic site of the viral polymerase mainly once viral minus strand DNA synthesis has been primed, as the viral polymerase undergoes conformational changes [25] that may allow FLG-TP to enter the catalytic site of the RT.

The mechanism of FLG inhibitory effect on DHBV reverse transcription is most likely to be due to a competition with the incorporation of dGTP, the natural substrate of the polymerase, and to the subsequent inhibition of the incorporation of following nucleotides in nascent viral DNA. Indeed, we observed that the IC₅₀ of FLG-TP for the WT DHBV polymerase was increased by 2.6-fold when the concentration of [α -³²P]dGTP was increased by 5-fold in the cell free assay. Furthermore, FLG-TP inhibited the incorporation of radiolabelled TTP suggesting that FLG-TP may be a competitive inhibitor of dGTP

incorporation in nascent viral minus-strand DNA and that it may terminate viral DNA chain synthesis, a mechanism previously observed with other nucleoside triphosphate analogs such as 3TC-TP and β LFd4C-TP [15, 24]. However, in our experimental conditions, neither FLG-TP nor ddGTP achieved to inhibit completely the DHBV RT activity (Figure 2). This lack of a complete inhibition of DNA primer synthesis may be explained by the fact that some of the polymerase polypeptides prime reverse transcription directly with TTP in the first position, as previously suggested [4]. Nevertheless, we cannot exclude that the incomplete inhibition of the DHBV RT activity may be due to the presence of endogenous dGTP despite our attempt to deplete dNTP from reticulocyte lysate by exclusion chromatography.

In Huh7 cells transiently transfected with WT HBV genome, FLG inhibited slightly more efficiently the HBV replication than PMEA, while 3TC displayed the most potent antiviral activity (Table 2). To summarize, we observed the following order of antiviral activity in Huh7 cells: 3TC >> FLG > PMEA. However, FLG-TP, 3TC-TP and PMEA-DP inhibitory activities on the elongation of viral minus strand DNA by DHBV polymerase in the cell free system were ranked in the following order: FLG-TP \approx PMEA-DP > 3TC-TP. In the latter system, the inhibition of the elongation of the viral minus strand DNA by a drug corresponds to its cumulative effects on the priming of the reverse transcription and on the termination of the DNA chain. 3TC-TP is not an inhibitor of the priming of the reverse transcription (21). Thus, its impact on the DNA chain elongation just relies on its effect as a DNA chain terminator, which could explain its weakest antiviral activity in the cell free system compared to PMEA-DP and FLG-TP. The discrepancies observed between the effects of the drugs on viral DNA synthesis in the cell-free system and the tissue culture may also suggest that the intracellular metabolism of the nucleos(t)ide analogs is important to take in consideration for the evaluation of drugs antiviral activity [22]. The weaker antiviral effect of FLG and PMEA in Huh7 cells compared to 3TC may be explained either by a decreased

entry and transport in the cell, by a poorer phosphorylation of these compounds or by a shorter intracellular half-life of the triphosphate form. Nevertheless, our results showed that FLG exhibits an interesting antiviral activity on the replication of WT HBV or DHBV. Moreover, we observed that FLG inhibited the intracellular DNA synthesis of WT HBV in Huh7 cells independently of viral genotypes (data not shown). This is in agreement with clinical studies that showed that antiviral response to other nucleoside analogs is, at least initially, independent of viral genotypes [29].

New findings came from the evaluation of FLG antiviral activity on the replication of HBV and DHBV drug-resistant mutants. The selection of drug-resistant HBV strains during prolonged clinical administration of 3TC or PMEA is becoming a major obstacle for the therapy of chronic HBV infection [2, 13, 27]. Currently, the L180M+M204V and the N236T HBV polymerase mutants are the more frequently observed in patients with 3TC or PMEA treatment failures, respectively. We therefore analyzed the cross resistance profile of FLG with 3TC-resistant (3TC-R) and PMEA-resistant (PMEA-R) mutants using both cell free assay allowing the expression of enzymatically active DHBV polymerases and Huh7 cells transfected with the genomes of these HBV mutants. Our results indicated that there is no significant difference between FLG inhibitory activity on the WT HBV or DHBV polymerase and on 3TC-R and PMEA-R mutants in both cell free assay and Huh7 cells (Table 1 and 2). We also assessed the antiviral efficiency of FLG against the DNA synthesis by HBV and DHBV polymerases harboring mutations conferring resistance to both 3TC and PMEA (3TC+PMEA-R mutant). Therapy combining 3TC and PMEA to treat patients infected with 3TC-resistant HBV strains [19] may select multiple drug-resistant mutant on the long-term. In a previous report, we showed that an HBV laboratory strain, harboring the L180M+M204V+N236T mutations within the polymerase gene, was not defective for replication and resistant to 3TC and PMEA [3]. Interestingly, we show here that FLG exhibits

a similar antiviral activity against the replication of this HBV mutant compared to WT HBV in transiently transfected Huh7 cells. Moreover, these results were consistent with those obtained with the *in vitro* expression system.

The study of the antiviral efficiency of FLG on HBV and DHBV drug-resistant mutants thus showed that FLG exhibited a similar inhibitory activity on WT, and on single and multiple drug-resistant HBV and DHBV polymerases in the two *in vitro* systems we used. Moreover, FLG inhibited more efficiently 3TC+PMEA-R polymerases than PMEA, whereas 3TC had no effect on these mutants. Altogether, our results suggest a lack of FLG cross-resistance against single 3TC- and PMEA- resistant mutants as well as against the double 3TC+PMEA-resistant mutant. These findings extend previous studies that showed that only purine analogs are active against the replication of L180M+M204V HBV mutant resistant to 3TC, whereas the same mutant is resistant to all known pyrimidine analogs [18, 31]. Similarly, the susceptibility to FLG of HIV-1 isolates, harboring mutation complex conferring resistance to thymidine analog, was weakly decreased *in vitro* compared to WT HIV-1 and no cross-resistance was observed (Zhang, H., B. Öberg, J. Harmenberg *et al.*, Abstr. 42nd Interscience Congress on Antimicrobial Agents and Chemotherapy, abstr. H-182, 2002). Consequently, FLG appears to be an efficient antiviral against both HIV and HBV resistant strains and may therefore be evaluated for the treatment of HIV/HBV coinfecting patients.

In conclusion, our results allowed us to gain new insight on the mechanism of action of FLG. The cross-resistance profile of FLG suggests that this compound could be envisaged as a new alternative drug for the treatment of chronic HBV carriers who have developed resistance to currently approved drug regimen. FLG may also be valuable for the design and evaluation of new combination therapies with other polymerase inhibitors for the treatment of chronic HBV infection to prevent or delay the emergence of drug-resistant mutants.

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LEGENDS TO FIGURES

Figure 1: Inhibitory activity of FLG on the activity of wild-type (WT) and different mutant (3TC-R, PME-A-R, 3TC+PME-A-R) DHBV polymerases.

A. Effect of FLG on the elongation of reverse transcription. Assays were performed for 30 min at 30°C with an equal volume of a reaction mixture containing 100 mM Tris-HCl pH 7.5, 30 mM NaCl, 20 mM MgCl₂, dATP, dCTP and dTTP (100 μM each), and [α^{32} P]-dGTP (0.165 μM ; 3,000 Ci/mm). The inhibition of [α^{32} P]-dGTP incorporation in the DHBV minus-strand DNA was performed with addition of increasing concentrations of FLG (0, 1, 5, 10, 50 and 100 μM respectively). Radiolabelled viral DNA covalently attached to polymerase (2 μl out of the 10 μl of the total reaction volume) is analyzed through 0.1% sodium dodecyl sulfate (SDS)-10% polyacrylamide gels and the dried gels were exposed to X-ray film. **B. Effect of FLG on the priming of the reverse transcription.** The experiments were the same than for the elongation reaction except that only [α^{32} P]-dGTP (0.165 μM ; 3,000 Ci/mm) was added to the reaction mixture. WT: wild-type; 3TC-R: L488M+M512V; PME-A-R: N544T; 3TC+PME-A-R: L488M+M512V+N544T.

Figure 2 : FLG-TP is a competitive inhibitor of the natural dGTP.

Assays were performed as described in Figure 1 except that dNTP mix is composed of dGTP and [α^{32} P]-TTP (0,165 μmol/L each). The incorporation of the radiolabelled TTP in viral minus strand DNA by the WT RT in absence or presence of increasing concentrations of the FLG-TP (indicated above the autoradiogram) is analyzed and quantified as described in Figure 1.

Figure 3: Activity of FLG-TP on the minus strand DNA chain termination of DHBV.

Assays were performed as described in Figure 1 except that the dNTP mix is composed of dGTP and [α^{32} P]-TTP (0,165 μmol/L each). The incorporation of radiolabelled TTP in viral minus strand

DNA by the RT in absence or presence of increasing concentrations of FLG-TP or ddGTP (indicated above the autoradiogram) is analyzed and quantified as described previously.

Figure 4: Activity of FLG on the replication of WT and drug-resistant mutants of HBV in transiently transfected Huh7 cells.

The antiviral activity of FLG was analyzed after transient transfection of Huh7 cells with either wild-type HBV genome (WT) or with 3TC-resistant (3TC-R; L180M+M204V), PMEA-resistant (PMEA-R; N236T) or 3TC+PMEA-resistant (3TC+PMEA-R; L180M+M204V+N236T) HBV genome cloned in pTriEXMod-HBV [6]. Sixty hours post-transfection, treatment with increasing concentrations of FLG (0, 1, 5, 10, 25 and 100 μ M) was started. Media were changed daily and drug administration was performed from day 3 to day 8 post-transfection. Cells were then lysed, HBV DNA was purified from intracellular core particles, separated on agarose gels and submitted to Southern Blot analysis. M.W.: molecular weight; Lin. HBV: linear DNA HBV.

TABLES

Table 1. Inhibitory activity of FLG-TP in comparison with 3TC and PMEAs on the priming and elongation activity of wild-type and mutant DHBV reverse transcriptases.

Drugs	WT Polymerase		3TC-R Polymerase		PMEA-R Polymerase		3TC+PMEA-R Polymerase	
	IC ₅₀ (μM)		IC ₅₀ (μM)		IC ₅₀ (μM)		IC ₅₀ (μM)	
	Priming	Elongation	Priming	Elongation	Priming	Elongation	Priming	Elongation
FLG-TP	> 100	4.9 ± 0.5	> 100	4.8 ± 0.8	> 100	6.7 ± 1.3	> 100	7.6 ± 1.9
3TC-TP	n.d.	8.0 ± 1.2	n.d.	> 100	n.d.	11 ± 1.4	n.d.	> 100
PMEA-DP	4.9 ± 0.4	4.6 ± 0.8	4.6 ± 0.5	4.4 ± 0.9	n.d.	39 ± 5.5	n.d.	52 ± 7.1

For each sample tested, the incorporation of radioactive dGTP in viral minus strand of DNA was estimated by quantitative dot assay. All results are first expressed in percentage of inhibition of the polymerase activity as compared to the reaction performed without any inhibitor. IC₅₀s were then deduced from the inhibition plot. This table shows the average IC₅₀ values obtained with three different experiments. n.d. : not determined. WT: wild-type; 3TC-R: L488M+M512V; PMEAs-R: N544T; 3TC+PMEA-R: L488M+M512V+N544T.

Table 2. Inhibitory activity of FLG, 3TC and PMEAs on the intracellular viral DNA synthesis of wild-type and mutant HBV laboratory strains in transiently transfected Huh7 cells.

Drugs	WT HBV Pol		3TC-R HBV Pol		PMEA-R HBV Pol		3TC+PMEA-R HBV Pol	
	IC ₅₀ (μM)	RF*	IC ₅₀ (μM)	RF*	IC ₅₀ (μM)	RF*	IC ₅₀ (μM)	RF*
FLG	9 μM ± 2.5	1.0	8 μM ± 3.8	0.9	13 μM ± 3.4	1.4	15 μM ± 6.8	1.7
3TC	0.5 μM ± 0.4	1.0	> 100 μM	> 200	0.8 μM ± 0.9	1.6	> 100 μM	> 200
PMEA	15 μM ± 3.4	1.0	12 μM ± 4.5	0.8	35 μM ± 5.5	2.3	> 100 μM	> 6.7

*: Resistance factor = IC₅₀ determined for mutant HBV / IC₅₀ determined for WT HBV.

WT: wild-type; 3TC-R: L180M+M204V; PMEAs-R: N236T; 3TC+PMEA-R: L180M+M204V+N236T.

Figure 1

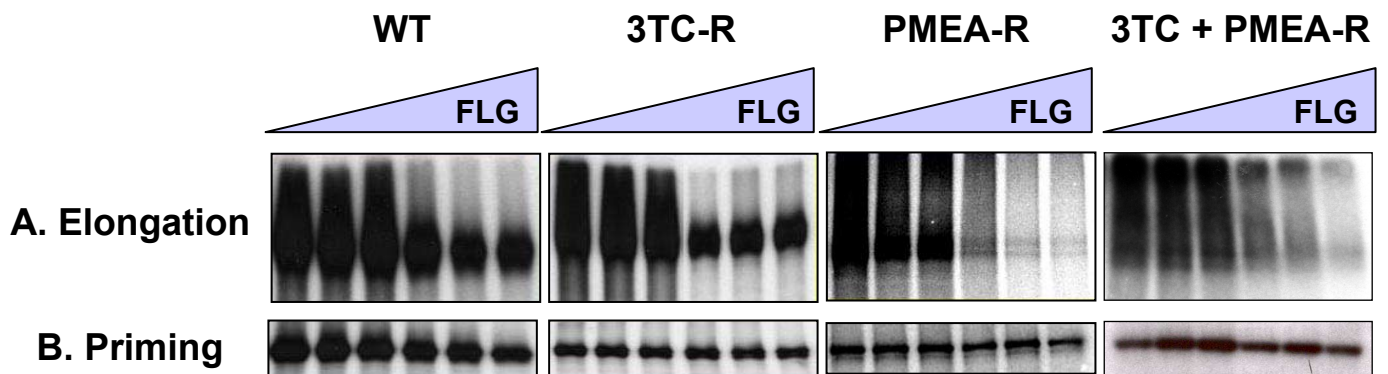


Figure 2

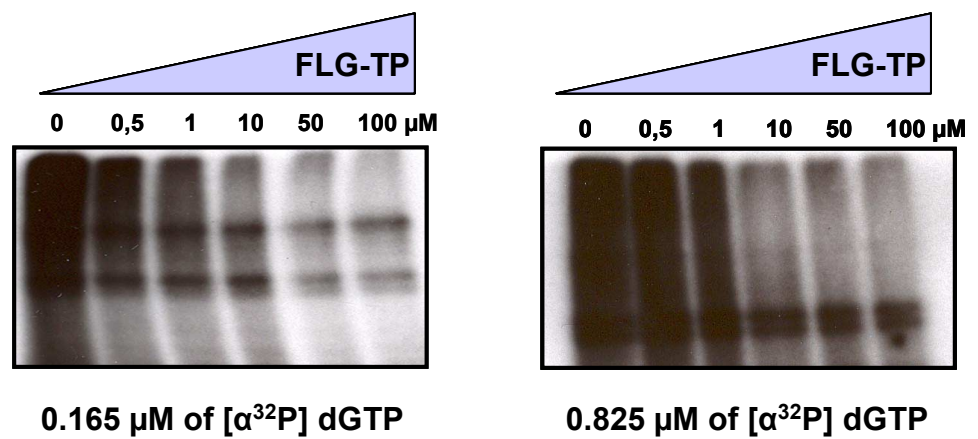


Figure 3

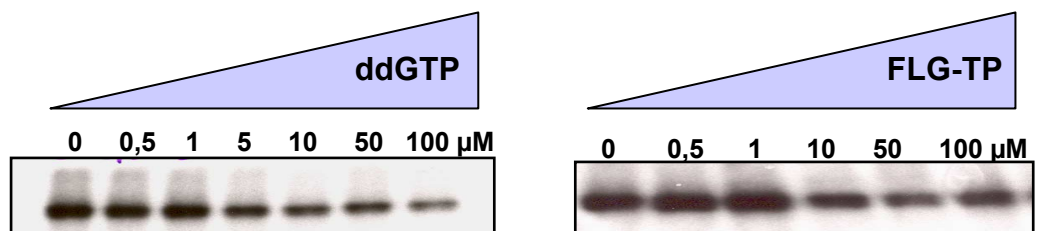


Figure 4

