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Inhibition of human hepatitis B virus (HBV) by a novel non-nucleosidic compound in a transgenic mouse model

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Abstract

BAY 41-4109 is a member of a class of heteroaryl-pyrimidines that was recently identified as potent inhibitors of human hepatitis B virus (HBV) replication. We have investigated the antiviral activity of BAY 41-4109 (methyl (R)-4-(2-chloro-4-fluorophenyl)-2-(3,5-difluoro-2-pyridinyl)-6-methyl-1,4-dihydro-pyrimidine-5-carboxylate) in HBV-transgenic mice (Tg [HBV1.3 fsX$^-$3′5′]). Bay 41-4109 was administered per os using different schedules (b.i.d. or t.i.d. for up to 28 days) and dosages ranging from 3 to 30 mg/kg. The compound reduced viral DNA in the liver and in the plasma dose-dependently with efficacy comparable to 3TC. In contrast to 3TC-treated mice, we found a reduction of cytoplasmic hepatitis B virus core antigen (HBcAg) in liver sections of BAY 41-4109-treated mice, which indicated a different mode of action. Pharmacokinetic studies in mice have shown rapid absorption, a bioavailability of 30% and dose-proportional plasma concentrations. We conclude that BAY 41-4109 is a new anti-HBV drug candidate.

Keywords: Antiviral activity; Animal models; Human hepatitis B virus; Transgenic mice

1. Introduction

Over 250 million people worldwide are persistently infected with human hepatitis B virus (HBV). As a direct consequence of chronic HBV infection, these people have an increased risk of developing liver cancer. Hepatitis B vaccines have been available for a long time, but HBV infection remains a global health problem, responsible for about 1.2 million deaths annually. New efficacious anti-HBV drugs have been developed recently.

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The efficacy of interferon (IFN) is partial and of limited duration, with less than 30% of the chronic carriers being treated with IFN responding to treatment. In addition, approximately 50% of those patients who respond to IFN therapy experience recurrence of viremia after cessation of the treatment (Fattovich et al., 1988; Thomas, 1998). The many side effects and lack of universal applicability of IFN have led to the investigation of other compounds. Nucleoside analogues such as Lamivudine [3TC, (−)-β-L-2′,3′-dideoxy-3′-thiacytidine] or Adefovir [9-(2-phosphonylmethoxyethyl)adenine] are now used or are in trials to establish a role in first line therapy (Poordad and Gish, 1999; DeClercq, 2001). However, despite the fact that anti-HBV drugs are capable of reducing viral loads very rapidly, the initial response is followed by a slow elimination of residual virus. In addition, the emergence of drug resistance during the slower phase of HBV elimination occurs frequently in 3TC-treated patients and limits a long termed treatment of HBV infection. Therefore, new and effective anti-HBV drugs are highly desired.

Here, we report that BAY 41-4109 (methyl (*R*)-4-(2-chloro-4-fluorophenyl)-2-(3,5-difluoro-2-pyridinyl)-6-methyl-1,4-dihydro-pyrimidine-5-carboxylate) is active in a transgenic mouse model of HBV infection.

2. Material and methods

2.1. HepG 2.2.15 cell assay for inhibition of HBV replication

Compounds were tested for their ability to inhibit HBV replication as described previously (Korba and Gerin, 1992). Briefly, HepG2.2.15 (Sells et al., 1987) cells were plated in 96 well plates and incubated together with dilutions of the compound at 37 °C and 5% CO_2. Medium was changed at day 4. Eight days after exposure to the compounds began, the supernatants were collected, the cells lysed and assayed for the presence of HBV-DNA by dot-blot hybridization and LumiImager quantification (Roche Diagnostics, Mannheim, Germany) analysis.

2.2. Compound

The structural formula of BAY 41-4109 is shown in Fig. 1. The compound was synthesized as described previously (Stoltefuss et al., 1998). 3TC ((−)-β-L-2′,3′-dideoxy-3′-thiacytidine) was purchased as tablets (Epivir®, Glaxo Wellcome, RTP, NC). The tablets were homogenized, and the compound was formulated as described below.

2.3. Animals

Breeding pairs for HBV-transgenic mice (Tg [HBV1.3 fsX$^-$3′5′]) were purchased from Professor Heinz Schaller (Centre for Molecular Biology, University of Heidelberg). These transgenic mice carry a frameshift mutation (GC) at position 2916/2917. The mice that were used for the experiments produced 10^7–10^8 virions per ml serum. The virus should be non-infectious in vivo as suggested by studies using X-mutated woodchuck hepatitis B virus (WHV) in the woodchuck (Chen et al., 1993; Zoulim et al., 1994). All animals were held under barrier conditions in a biosafety level 2 facility according to the permits.

2.4. Treatment of transgenic mice

The HBV-transgenic mice were checked for HBV-specific DNA in the serum by PCR prior to the experiments. Each treatment group consisted of five male and five female animals approximately 10 weeks age. Compounds were formulated as a suspension in 0.5% Tylose (Hoechst,

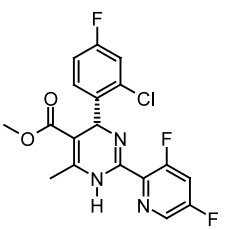

Fig. 1. Structural formula of BAY 41-4109. In experiments using HepG2.2.15 cells, the inhibitory concentration 50 (IC_{50}) of BAY 41-4109 against HBV was estimated to be 53 nM and against HepG2.2.15 cells (CC_{50}) to be 7 μM.

Frankfurt, Germany) and administered per os to the animals two times/day for a 28 day period unless otherwise indicated. The 0.5% Tylose served as a placebo. Six hours after the last treatment, the animals were sacrificed and livers were removed and immediately frozen for subsequent analysis. Blood was obtained by cardiac puncture of the anesthesized animals.

2.5. Analysis of HBV-DNA in the livers of HBV-transgenic mice by DNA-dot-blot

Fifty milligrams of frozen tissue was minced and digested in 0.5 ml proteinase K buffer (10 mM Tris–HCl, pH 8.0; 100 mM NaCl; 0.5% SDS; 25 nM EDTA) with 0.1 mg/ml proteinase K overnight at 56 °C. The nucleic acids were extracted using the phenol/chloroform/isoamyl alcohol procedure, precipitated with ethanol and resuspended in 100 ml of 10 mM Tris buffer (pH 8.0). DNA concentrations were estimated using the formula $OD_{260} \times 50 = mg/ml$ and with examination of each sample by gel electrophoresis and ethidium bromide staining.

Dot-blot hybridization was carried out with a DIG-labelled, random primed (DIG-labelling and detection kit; Roche Mannheim, Germany) full length HBV-DNA probe prepared according to the manufacturer's instruction using a standard dot-blot apparatus (Dunn, Germany). Briefly, 200 μl of DNA (10 μg) was spotted onto a nylon membrane (Roche, Mannheim, Germany) soaked four times for 3 min each time with Soak I (0.5 N NaOH; 1 M NaCl), two times with Soak II (3 M NaCl; 0.5 M Tris–HCl, pH 7.4), baked at 120 °C for half an hour, pre-hybridized in a standard hybridization buffer (5 × SSC, *N*-lauroylsarcosine, 0.1% w/v; SDS, 0.02%; blocking reagent, 1 × and 100 μg/ml fish sperm DNA) without a probe for 30 min at 60 °C and hybridized over night in the presence of a probe (concentration of probe: 20–40 ng/ml; total volume 200 ml). The filter was subsequently washed at 64 °C in 4 × SSC/0.1%SDS, 2 × SSC/0.1%SDS and 1 × SSC/0.1%SDS for 10 min, respectively. The immunological detection was carried out using the CDP-Star system (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. Briefly, the membrane was incubated for 30 min in 100 ml blocking solution and afterwards for another 30 min in 20 ml/100 cm^2 antibody–conjugate-solution (1:20 000). Subsequently, the filter was washed twice for 15 min in washing buffer and equilibrated for 5 min in 20 ml detection buffer. For chemiluminescent detection, 1 ml of a 1:100 diluted CDP-star-solution was pipetted into a hybridization bag, the filter was transferred into the solution-containing bag and incubated for 5 min. The CDP-star-solution was then removed from the bag and the filter was exposed for chemiluminescence detection and quantification in a LumiImager (Roche Diagnostics, Mannheim, Germany).

2.6. Analysis of HBV-specific DNA in plasma of HBV-transgenic mice

DNA was prepared from 100 μl of plasma that was collected in EDTA tubes using the QIAamp Blood Kit (Qiagen, Hilden, Germany) as described by the manufacturer with an additional concentration step (ethanol precipitation using 1 μg calf thymus DNA (Sigma Chemical Co., St. Louis, MO) as a carrier). The pellet was resolved in 20 μl of 10 mM Tris buffer (pH 8.0). Two microliter, were used for quantitative PCR analysis using the ABI PRISM™ 7700 Sequence Detection System (PE Applied Biosystems, Foster City, CA). The PrimerExpress™ software (PE Applied Biosystems) was used to select the primers:

ayw-570f (sense)	5′-CTGTACCAAACCTTCG-GACGG-3′
ayw-670r (antisense)	5′-AGGAGAAACGGGCTGA-GGC-3′
and the probe:	
ayw-613t	5′-CCATCATCCTGGGCTT-TCGGAAAATT-3′

Plasma DNA was amplified in a 50 μl reaction volume containing 1.4 mM of each dNTP, 4.75 mM $MgCl_2$, 15 pmol of each primer and the probe, 5 μl 10-fold PCR buffer (all PCR reagents

were used from the TaqMan core reagent kit; Perkin–Elmer/Roche Molecular Systems Inc., Branchburg, NJ), 1.25 U Taq DNA polymerase and 0.25 U Amp Erase. After an initial denaturation step (95 °C for 10 min), samples were subjected to 40 rounds of denaturation (95 °C for 30 s) and annealing/extension (56 °C for 1 min). Analysis of the products was carried out using the ABI PRISM™ 7700 Sequence Detection System standard software.

2.7. Histological and immunohistological analyses

Liver specimen from one or two lobes were fixed in 4% formaldehyde solution overnight at room temperature and embedded in paraffin. Two paraffin sections (5 μm) were prepared from each liver and stained with hematoxylin and eosin. In order to perform immunohistological analyses, paraffin-embedded sections were deparaffinized and rehydrated. Endogenous peroxidase activity was quenched with 3% H_2O_2 for 20 min. Non-specific binding was blocked with normal sheep serum. Subsequently, the sections were incubated for 30 min at room temperature with a 1:500 diluted polyclonal rabbit antibody against HBcAg (Dako, Hamburg, Germany). The following steps were performed according to the avidin–biotin-procedure using the Vectastain ABC kit as described by the manufacturer (Vector Laboratories, Inc. Burlingame, CA). The immunoreaction was visualized by the addition of 3.3 diamino-benzidine tetrachloride and hydrogen peroxide. The livers were counterstained with hematoxylin. Non-immune rabbit serum served as a control. The number of positively labeled nuclei and the amount of cytoplasmic immunostain was estimated and scored (grade 1–3) for each mouse (see footnote in Table 2).

2.8. Pharmacokinetics

Blood was obtained after intravenous and oral administration at various time points (three animals per time point) up to 24 h post dose. After centrifugation, plasma was precipitated with acetonitrile and BAY 41-4109 was determined in the supernatant by LC/MSMS using Turbo Ion Spray Ionisation. Pharmacokinetic parameters were calculated from plasma concentrations with KINCALC, a validated in-house calculation program.

Table 1
Pharmacokinetic parameters of BAY 41-4109

PK parameter	Mouse	Rat	Dog	Human
Clearance (l/h kg)	4.01	1.75	0.72	0.46[a]
$t_{1/2}$ i.v. (h)	≤ 1	2	5	4
Bioavailability (%)	31	58	58	48

[a] Predicted from the allometric species scaling approach according to H. Boxenbaum, 1982 (Journal of Pharmacokinetics and Biopharmaceutics, Vol. 10, No. 2).

2.9. Statistical analysis

HBV-DNA reduction was analyzed by using *Variance analysis* with subsequent post-hoc comparison of means (STATISTICA, StatSoft, Tulsa, Oklahoma).

3. Results

3.1. BAY 41-4109 reduces HBV-specific DNA in HBV-transgenic mice

In experiments using HepG2.2.15 cells (Sells et al., 1987) the inhibitory concentration 50 (IC_{50}) of BAY 41-4109 (Fig. 1) against HBV was estimated to be 53 nM, the IC_{50} of 3TC against HBV was estimated to be 220 nM.

The pharmacokinetic data showed that even low dosages are sufficient to exceed in vitro IC_{50} levels in plasma (see below and Table 1). However, since the compound displayed a relatively short half-life of 2 h in mice, we decided to administer the compound twice a day (b.i.d.).

We have used a strain of HBV-transgenic mice (Tg [HBV1.3 fsX$^-$3′5′]) that carries a frameshift mutation (GC) at position 2916/2917 (Schaller and Kuhn, unpublished). Viral DNA from mouse plasma was estimated by quantitative real time PCR. We have used dilutions of known amounts of HBV-DNA from human plasma as a control. The mice were pre-selected and only mice that expressed 10^7–10^8 genome equivalents per ml

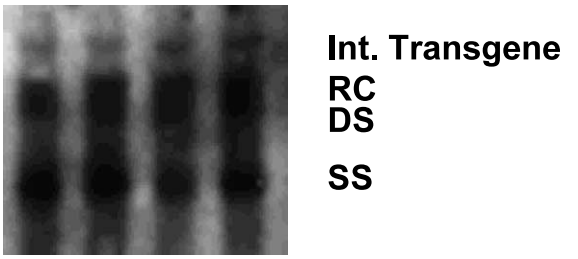

Fig. 2. Forms of HBV-DNA in livers of the transgenic mice. Twenty micrograms liver-DNA from $n=4$ animals was RNAse-treated before gel electrophoresis. Bands corresponding to the expected size of the integrated transgene and the forms that appear during active HBV replication, relaxed circular (RC), double stranded linear (DS), and single stranded (SS) HBV-DNA are indicated. DNA amounts were normalized by OD_{260} and agarose gel electrophoresis.

plasma were used in the experiments. The virus should be non-infectious in vivo according to studies using X-mutated WHV in the woodchuck (Chen et al., 1993; Zoulim et al., 1994). Viral replication and viral morphogenesis are not afflicted by this mutation. The viral replication was investigated by Southern blot analysis of replicative intermediates of HBV-DNA in these mice. All HBV-DNA-intermediates that are detected in natural infections have been found in the transgenic mice, too (Fig. 2). Morphological integrity of the virions was investigated by electron microscopy and typical HBV virions have been detected (not shown).

As depicted in Fig. 3, administration of BAY 41-4109 reduced HBV more effectively than 3TC in liver and plasma of the HBV-transgenic mice. The results are reproducible and are not gender-specific as the antiviral activity was observed to be the same in female mice (not shown). A good dose-response was obtained with BAY 41-4109 in the livers that is indicated by a significant difference between the 30 and the 15 mg/kg dosage groups. The antiviral effects were more pronounced in the plasmas. However, 3TC did not show significant effects at the dosage and schedule used due to high variability.

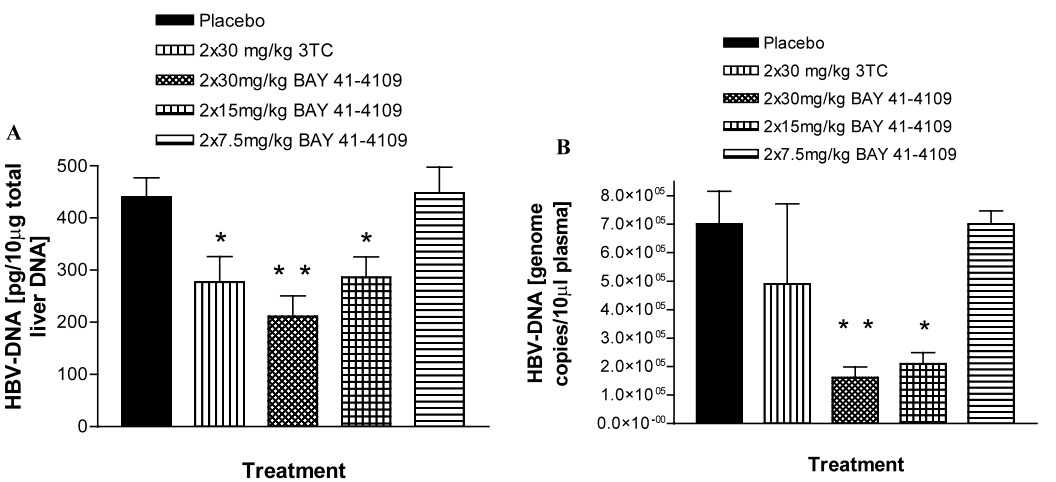

Fig. 3. Effect of BAY 41-4109 or 3TC-treatment in HBV-transgenic mice ($n=10$). The compounds were administered at the indicated dosages twice a day for 28 consecutive days. (A) HBV-DNA specific signals in liver samples were quantified using dot-blot hybridization. BAY 41-4109 was more effective than 3TC in reducing HBV-specific DNA in the livers ($P<0.05$). Whereas the antiviral activity of BAY 41-4109 was significant at 30 and 15 mg/kg ($P<0.05$), no antiviral effect was observed at the 7.5 mg/kg dosage. (*$P<0.05$, **$P<0.01$, Variance analysis with subsequent post-hoc comparison of means (LSD-test). (B) Quantitative real time PCR was used to detect HBV in the plasma of the transgenic mice. The antiviral effect of BAY 41-4109 was more pronounced than in the liver. No significant antiviral effects were obtained when 3TC was administered at a dosage of 30 mg/kg. In contrast, significant antiviral effects were detected for BAY 41-4109 at 15 and 30 mg/kg. (*$P<0.05$, *Variance analysis* with subsequent post-hoc comparison of means (LSD-test), **$P<0.05$ to *).

3.2. BAY 41-4109 reduces hepatitis B virus core antigen (HBcAg) in livers of HBV-transgenic mice

Immunohistological analyses of the livers were performed in order to assess the influence of the treatment to HBV core antigen (HBcAg). In untreated and control animals that received a placebo, HBcAg was detected in the liver cell nuclei and the cytoplasm of centrilobular hepatocytes. Cytoplasmic HBcAg decreased to undetectable levels throughout the liver in BAY 41-4109-treated animals despite the fact that the hepatocytes were cytologically normal. A typical result is shown in Fig. 4. Compared to the vehicle control group, a reduction of cytoplasmic HBcAg was observed in both female and male mice treated with BAY 41-4109. The number of labeled nuclei was reduced in 3TC-treated females but not in males indicating a gender-specific effect (Table 2).

3.3. PK profile of BAY 41-4109

BAY 41-4109 is a high clearance drug in mice. However, clearance decreases with increasing size of the animal species. After oral administration, absorption is fast ($t_{max} = 0.17$ h) in mice and rats. Absorption is also fast in dogs ($t_{max} = 0.8$ h). This indicates that the bioavailability is determined by the clearance of the compound and that the absorption of BAY 41-4109 is likely to be complete. Oral bioavailability of BAY 41-4109 is approximately 30% in mice and about 60% in rats and dogs. Human clearance and volume of distribution ($V_{ss} = 2.9$ l/kg) were predicted from the allometric species scaling approach. Half-life was calculated according to the equation $t_{1/2} = V_{ss} \ln 2/CL$. Based on the assumption that absorption is complete, bioavailability was predicted according to the equation $F_{max} = 1 - (CL_{blood}/Q_H)$.

4. Discussion

The purpose of our study was the characterization of a novel class of heteroaryl-pyrimidines in a relevant animal model of HBV replication. Several animal models have been described. Whereas the duck hepatitis B virus (DHBV) (Mason et al., 1980) has been the hepadnavirus of choice for molecular studies of hepadnaviruses, the woodchuck—woodchuck hepatitis B virus (WHV) (Summers et al., 1978) model provides the best studied in vivo system for investigating the pathogenesis of virus-caused hepatocellular carcinoma (HCC) (Tennant, 1999). Moreover, this model is broadly accepted for investigating novel antiviral agents and strategies (Mason et al., 1998; Moraleda et al., 1997; Tennant et al., 1998).

More recently, transgenic mouse models have been developed (Nagahata et al., 1992; Guidotti et al., 1995). These mice show virus titers that are comparable to that found in acutely infected human patients. Moreover, HBV-transgenic mice are useful for the investigation of novel compounds that act against steps in the viral replication cycle (Nagahata et al., 1992; Morrey et al., 1999). Transgenic mice cannot be used to investigate either the early steps of infection or the clearance of the virus from the body.

A sustained reduction of viral load is a prerequisite to prevent the emergence of mutant drug-resistant viruses (Marcellin et al., 1997) and to clear viral infection by the immune system. We did not monitor HBV e or s antigens in the plasmas. These HBV surface antigens are commonly used as surrogate markers for measuring viral load. Although these are important HBV markers in human patients, expression of HBV surface antigens in transgenic mice was found to be regulated by sex steroids (Farza et al., 1987). In addition, the HBsAg titers vary with age whereas HBV-DNA content does not (not shown). Morrey et al. (1999) did not find a correlation of serum DNA with either age or serum antigens. These authors attributed this finding to the PCR technique used in their investigation. However, the quantitative real time PCR used in our assays was very robust. Therefore, we have used viral DNA as a marker for viral load, viral replication and antiviral response to treatment. Analyses of Southern blots from total liver DNA using the LumiImager System (Roche Diagnostics, Mannheim, Germany) as described in Section 2 showed that the HBV-

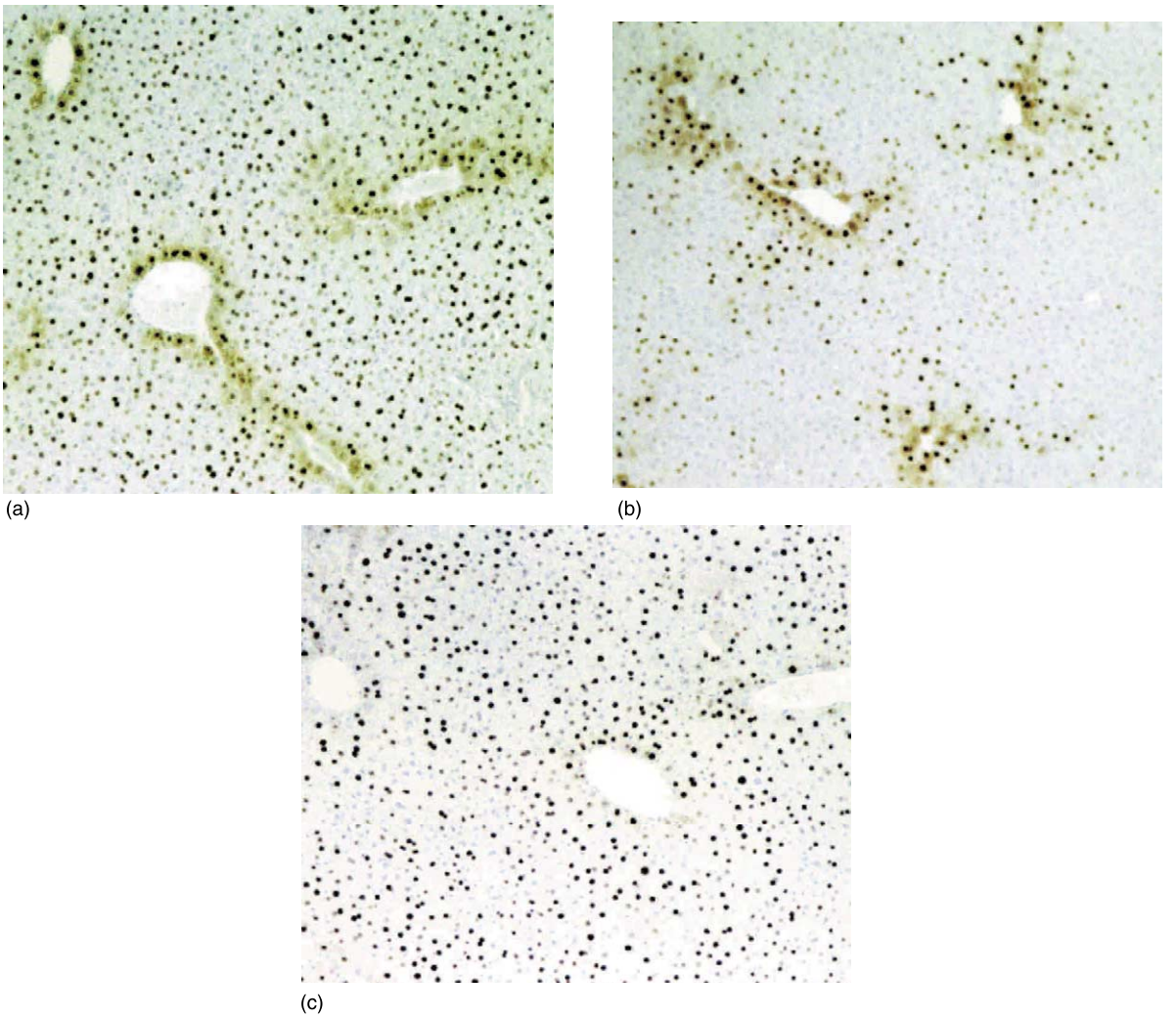

Fig. 4. Immunohistological detection of hepatitis B virus core antigen (HBcAg) in the livers of HBV-transgenic mice. Male and female mice ($n = 5$/gender and group) were treated with 30 mg/kg BAY 41-4109 or 3TC t.i.d. for 20 consecutive days, the livers were removed and stained for HBcAg (nuclear HBcAg: black dots; cytoplasmic HBcAg: diffuse brown stain in cells surrounding central veins). (A) Male animals treated with the vehicle control (placebo) showed grade 3 nuclear staining and grade 2 or 3 cytoplasmic staining. In females, the nuclear and cytoplasmic immunostain was scored grade 3. The picture shows a liver section of a female mouse. (B) After treatment with 3TC nuclear and cytoplasmic HBcAg was similar in male mice when compared to placebo-treated mice. In females, the number of HBcAg positive nuclei appeared to be reduced to grade 2. However, the cytoplasmic HBcAg content was similar in placebo-treated females and females treated with 3TC. A liver section of a female mouse is shown here. (C) The cytoplasmic HBcAg content was reduced to a high degree or even absent in male and female mice after administration of BAY 41-4109. No influence of BAY 41-4109 treatment on the nuclear staining pattern was observed. A liver section of a female mouse is shown.

transgene accounts for approximately 5% of the HBV-specific signal in livers. Approximately 95% of the signal represent de novo synthesized HBV-DNA including replicative intermediates and virion-DNA. In the plasma, the transgene-effect was presumably even lower since all purification steps were carried out carefully in order to prevent cellular contamination of the plasma.

We have investigated approximately 20 compounds of this class in vivo and have identified BAY 41-4109 as the most potent compound. The structure–activity-relationship of this class of compounds is described in detail elsewhere (Goldmann et al., 2001). BAY 41-4109 reduced hepadnaviral DNA more effectively than 3TC in livers and plasma of the mice. As mentioned above, the transgenic mice produce HBV at a high level. Since the compound displayed a short half-life in mice, we have initially decided to treat the animals three times a day. However, in subsequent experiments we have obtained comparable antiviral effects employing a b.i.d. (twice a day) schedule. The duration of the treatment was estimated from preliminary studies that were carried out to investigate the antiviral effect over the time-course of treatment. Good antiviral effects were observed with both 3TC and BAY 41-4109 after 20 days of treatment and maximal antiviral effects after 28 days of treatment using BAY 41-4109 and 3TC. It has recently been described that 3TC treatment reduced plasma HBV-DNA in HBV-transgenic mice by more than 1.0 log 10 (Morrey et al., 1999). However, both the semi-quantitative PCR technique that was used by these authors to monitor the antiviral effects and the different strain of HBV-transgenic mice that had substantially lower pre-treatment HBV titers are responsible for the differences in 3TC activity observed in both models.

We did not conduct clearance or rebound studies since these mice are not suitable models to address these questions because of their continuing replication of HBV from the transgene. The antiviral effects of BAY 41-4109 in plasmas did correlate with those in the livers of treated mice indicating that the antiviral effect is indeed related to the inhibition of viral replication. The quantity of all replicative intermediate DNA species was reduced after treatment to the same extent in livers of mice after treatment with both 3TC and BAY 41-4109. The HBV-DNA replication occurs inside the novel formed capsids in the cytoplasm (reviewed by Ganem and Varmus, 1987). The role of nuclear staining in these mice remains speculative and could be either artificial or de-novo synthesized (Guidotti et al., 1995). However, nuclear nucleocapsids do not contain viral DNA, i.e. they are empty (Guidotti et al., 1995). The fact that we

Table 2
HBcAg content in the livers on HBV-transgenic mice: summary incidence of gradings ($n = 5$ animals/group and gender, male placebo-treated mice: $n = 6$).

Subcellular localization of HbcAg	Gender	Grade of HBcAg stain	Placebo	3TC	BAY 41-4109
Nucleus	Male	1	–	–	–
		2	–	1	–
		3	6	4	5
	Female	1	–	2	–
		2	1	1	–
		3	4	2	5
Cytoplasm	Male	1	–	–	4
		2	2	–	1
		3	4	5	–
	Female	1	–	–	2
		2	–	2	3
		3	5	3	–

Scores of anti-HbcAg stain: *Nuclei*: grade 3: the majority of nuclei in the liver specimens revealed intense immunostaining; grade 2: the number of positive nuclei was slightly reduced predominantly at the periphery of the liver lobule; grade 1: further reduction of positive nuclei was observed. *Cytoplasm*: grade 3: strong staining of numerous hepatocytes was observed around the central veins; grade 2: the cytoplasmic staining was reduced; grade 1: a further reduction or even absence of cytoplasmic immunostaining was observed. The histological slides were cross-checked by an independent pathologist and the results confirmed.

have observed significant reduction of cytoplasmic HBcAg after BAY 41-4109 treatment but not after 3TC treatment indicates that the mechanism(s) of the antiviral activity of BAY 41-4109 differ from that of nucleosides. The antiviral activity could either be due to direct inhibition of the replication machinery and/or to inhibition of the viral assembly process. More detailed analyses of the mode of action will be described elsewhere (Deres et al., submitted).

In summary, we have characterized a novel non-nucleosidic compound with an anti-HBV activity that is different regarding potency and mode of action from 3TC in HBV-transgenic mice. The compound displays a fast absorption and a good oral bioavailability in mice and our pharmacokinetic studies in rats and dogs support the assumption that this compound will have a good profile in humans. In addition, we did not observe any signs of toxicity in our studies. Additional studies that address aspects such as tolerability, combinations with other drugs and the mechanism of action have to be conducted. We conclude that BAY 41-4109 represents a promising drug candidate.

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