VIRAL DYNAMICS IN CHRONIC HEPATITIS B PATIENTS

L.M.M. Wolters

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VIRALE DYNAMIEK BIJ PATIËNTEN MET CHRONISCHE HEPATITIS B

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Copromotor:	Dr. R.A. de Man

Voor papa en mama

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1 A

GENERAL INTRODUCTION

L.M.M. Wolters, R.A. de Man

Department of Gastroenterology & Hepatology, University Hospital Rotterdam,
The Netherlands

A chronic hepatitis B infection (HBV) is a potentially progressive liver disease which affects around 350 million people world-wide with an annual mortality rate of around 1 million people. (1). Together with malaria and tuberculosis it is among the top three of most prevalent infectious diseases. The evolution of this viral infection is dependent on the activity of the inflammation which is induced by both host and viral factors. HBV may be responsible for death due to liver cirrhosis or hepatocellular carcinoma (HCC) with a lifetime risk of 40-50% in men and 15% in women (2). Universal vaccination which is common practice in over 80 countries world-wide (3) has the potential to eradicate this viral disease completely and prevent HCC. Although vaccination in highly prevalent areas is much cheaper compared to the western world, universal vaccination still generates substantial costs per prevented infection. Screening for the presence of HBV in pregnant women is a more low-scale intervention which is capable of protecting selected neonates by post-natal immunization. This program is capable of protecting infants who otherwise run a great risk to become chronically infected (4). However, since at present there is a reservoir of millions of people who are chronically infected, we are in need of effective antiviral therapies. The main initial goal of antiviral therapy should be induction of HBeAg seroconversion and normalization of serum transaminases. This results in a low infective individual with a quiescent disease in whom the chance of further liver damage has been minimized (5,6).

Since patients who express active viral replication (as shown by HBeAg positivity and high levels of HBV DNA) and/or elevated serum transaminases are at the highest risk to develop complications of the liver disease, therapy should primarily be aimed at this subgroup of chronically infected patients.

More than a decade of intensive research has resulted in two registered therapies which both have limitations. Alpha interferon was the first drug which has proved to cause induction of HBeAg seroconversion in 30-40% of patients (7-9). Elevated baseline ALT and lower levels of HBV DNA at start of therapy are predictors of a positive response to alpha interferon treatment (10). However, the majority of HBV infected patients will not benefit from this therapy which is also hampered because of side-effects (11). Extensive research in Human Immunodeficiency Infected (HIV) patients has resulted in the production of synthetic nucleoside analogues that are capable of inhibiting viral replication. Lamivudine, 2'3'-dideoxy-3'-thiacytidine, is the only registered compound which has proved to be effective in both HIV and HBV infected patients. Lamivudine is an obligatory chain terminator that has been applied in several large studies causing only few, non-serious, side-effects. It has recently been registered for the treatment of chronic liver disease related to HBV (11-14). It has proved to be capable of reducing HBV DNA to below levels of detection in insensitive assays in the majority of patients after 6 months of therapy (11). The decline of virus is accompanied by normalization of serum transaminases and improvement of histology in the majority of patients with pronounced viral reduction (12). Even though many patients benefit from this therapy, some disadvantages become apparent after several months of lamivudine treatment.

First of all, data on patients who were treated with lamivudine for up to 2 years indicate that a part of patients never reaches HBV DNA negativity as measured with insensitive assays (11,13,15). Secondly, prolonged lamivudine therapy is hampered by a time-dependent emergence of YMDD mutations of the polymerase genome of the HBV (16-17). Lastly, even though lamivudine induces HBeAg seroconversion in 17% and 27% of patients after 1 and 2 years of therapy (13), it is durable in only a minority of patients (19). The rebound of viral replication which may induce severe liver disease (20) is due to residual virus that is still

present in the liver. An important part of this residual virus (cccDNA) is resistant to lamivudine therapy (21,22). Prolongation of therapy could theoretically result in viral eradication because of natural turn-over of infected hepatocytes. However, most patients will become resistant before complete viral eradication has been accomplished.
Because alpha interferon and lamivudine can not be applied universally for all chronic hepatitis B patients, new approaches should be explored. Most probably, recovery from the HBV infection requires a combined action of inhibition of viral replication and turnover of infected hepatocytes, either by the immune system or through induction of host defense mechanisms by antiviral drugs. To be able to evaluate new therapies however, it is important to describe more accurately mechanisms responsible for bypassing the present therapy.
The objectives of this study were:
*To evaluate the mechanisms of diminished response to lamivudine.
*To evaluate lamivudine therapy and new nucleoside analogue therapies by means of mathematical modeling in single drug as well as in combination therapy.

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1 B

NUCLEOSIDE ANALOGUES FOR CHRONIC HEPATITIS B

L.M.M. Wolters[1], H.G.M. Niesters[2], R.A. de Man[1]

Department of [1]Gastroenterology & Hepatology and [2]Virology,
University Hospital Rotterdam, The Netherlands

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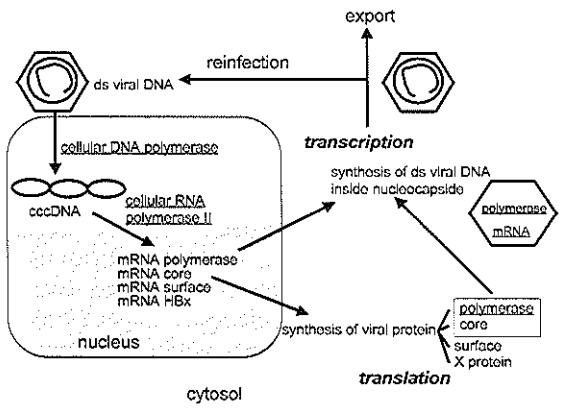
Summary

Hepatitis B virus replicates inside the hepatocyte through an intermediate step of reverse transcription mediated by the viral polymerase. We describe 5 nucleoside/nucleotide analogues that interfere with the replication mechanisms of the hepatitis B virus. The resemblance of nucleoside analogues to natural nucleosides may lead to direct cytotoxicity. Therefore, antiviral activity should always be interpreted in the light of cellular toxicity.
In addition, prolonged therapy with a nucleoside analogue may induce mutations in the viral polymerase causing structural and configurational changes of the polymerase resulting in a decreased affinity for the nucleoside analogue. Subsequently, the mutated virus is capable of renewed replication during continued antiviral pressure of the nucleoside analogue.
The best antiviral strategy in the future is probably combination therapy, either with several nucleoside analogues or with a nucleoside analogue and interferon.

Introduction

Replication of the hepatitis B virus
Hepatitis B virus (HBV) is a partially double stranded DNA virus converted to covalently closed circular DNA (cccDNA) after uptake into the hepatocyte (Fig 1).

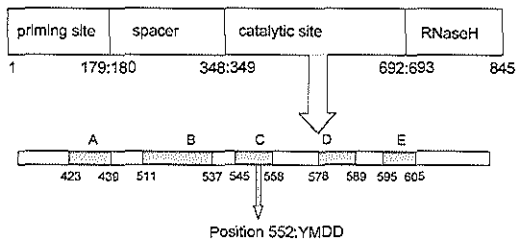
Fig 1 Hepatitis B viral replication inside the hepatocyte.



This conversion is most likely catalyzed by cellular DNA polymerases (1, 2). The cellular RNA polymerase II regulates transcription of the cccDNA template and forms a greater than genome-length mRNA. After translation into the viral polymerase, viral replication continues inside the nucleocapsid in the cytosol. This polymerase consists of three functional domains for priming, reverse transcription and RNAse H activity (Fig 2). There is a fourth domain, called the spacer, whose function is not known.

The polymerase initiates reverse transcription of mRNA to the (-) DNA strand while adhering to the encapsidation signal of the mRNA. The hydroxyl group of tyrosine 96 within the priming domain of the polymerase is the acceptor of the first nucleotide of the (-) DNA strand. After covalent binding of three nucleotides to tyrosine at the 5' terminus, the polymerase complex is dissociated from the mRNA and translocated to a complementary sequence in the direct repeat region 1 (DR1) near the 3' end of the mRNA. The polymerase adheres to free nucleotides inside the nucleocapside and incorporates them in the growing (-) DNA chain. Meanwhile, RNase H digests the RNA within the RNA-DNA hybrid structure leaving a small part of the RNA chain at the 5' terminus (approximately 17-18 nucleotides). This RNA oligomer acts as the primer for the formation of the (+) DNA chain.

Fig 2 HBV genotype A. The HBV polymerase consists of four domains. The YMDD sequence in the C region of the catalytic domain is the main site of interaction with nucleoside analogues

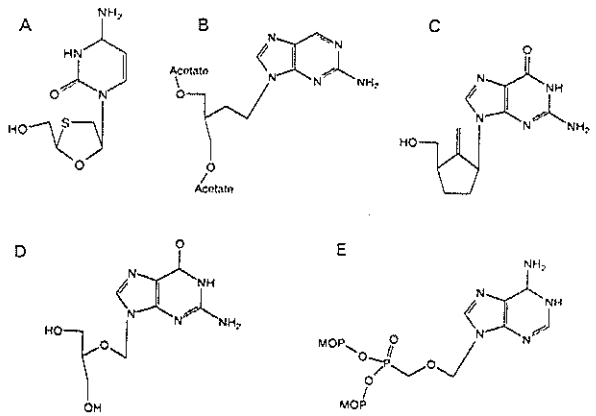


The elongation of this chain is again mediated by the viral polymerase and is only partially generated. The nucleocapsids can either be retransported into the nucleus or they can leave the cell as a complete Dane particle after acquiring the outer envelope membrane.
The various steps of HBV replication provide opportunities for antiviral drugs to interact with the virus. Nucleoside analogues are chemically synthesized drugs (natural D- or unnatural L-configuration) (3), which are highly comparable to natural nucleotides. This makes them suitable for inhibition of viral replication but, due to possible non-selective inhibition of the replication of cellular DNA, also makes them potentially toxic.

Mechanism of action and efficacy of nucleoside analogues

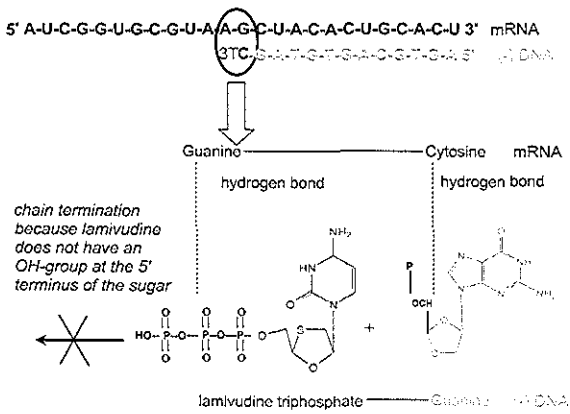
Nucleoside and nucleotide analogues can be subdivided into the group of purines (adenosine and guanine) and the group of pyrimidines (thymidine and cytosine) which they mimic: lamivudine (cytosine analogue), famciclovir, entecavir and ganciclovir (guanosine analogues) and the nucleotide adefovir dipivoxil (adenosine analogue). The purine analogues (guanine and adenine) are modified cyclic or acyclic sugar configurations whereas the pyrimidine analogue lamivudine has an unnatural L-configuration. The group of antiviral compounds will hereafter, for the sake of simplicity, be referred to as nucleoside analogues.
The main action of the nucleoside analogues is interference with the elongation of viral chains through competitive inhibition with the viral polymerase. The affinity of the nucleoside analogue for the viral polymerase is expressed by the K_i/K_m ratio. K_m is the constant, which describes binding of the natural nucleoside to the polymerase. K_i expresses the affinity of a nucleoside analogue for a polymerase. The nucleoside analogue shows higher affinity for the viral polymerase than for the natural nucleoside if the K_i/K_m ratio of the viral polymerase is less than 1. A K_i/K_m ratio less than 1 indicates that a higher concentration of the natural nucleoside with respect to the nucleoside analogue is needed for the two to be incorporated in the viral chain equally. After incorporation of the nucleoside analogue, chain termination of either the (-) or (+) DNA strand may be accomplished.

Fig 3 **A** Lamivudine; **B** Famciclovir; **C** Entecavir; **D** Ganciclovir; **E** Adefovir dipivoxil.



Lamivudine, the negative enantiomer of 2'3'-dideoxy-3'thiacytidine (Fig 3A) is metabolized to its mono-, di- and triphosphate inside the hepatocyte (4). The rate-limiting step is the conversion of the di-phosphate to the active tri-phosphate, the latter comprises only 15% of intracellular lamivudine metabolites (4) and can be incorporated in both DNA strands. Since the Ki/Km ratio for lamivudine is >1, the incorporation of the natural cytosine is favored over lamivudine (5). Because of the structure of this analogue, which contains a sulfur atom instead of an OH-group at the 3' terminus, the viral chain cannot be elongated and viral replication will stop (Fig 4).

Fig 4 Interference of lamivudine with HBV replication



The guanine analogues famciclovir, entecavir and ganciclovir are also metabolized to the active triphosphate. However, in contrast to lamivudine, the concentration of the active entecavir triphosphate is the highest compared to the mono- and diphosphate metabolites (6). In this respect, the drug profile is optimal. Moreover, guanine analogues are capable of inhibiting both the priming reaction of the polymerase (7-9), because they bind to the tyrosine at the priming site of the polymerase (9), and elongation of both DNA strands of the virus. These guanine analogues have a 3'hydroxyl group used for chain elongation but due to the structural changes, which they induce, chain elongation terminates two or three nucleotides downstream (10-13). Also, the relatively low intracellular concentration of guanine nucleotides may facilitate the incorporation of these nucleoside analogues.
Famciclovir itself does not have antiviral activity but is deacytylated and oxidized to penciclovir (Fig 3B). Intracellularly, penciclovir is phosphorylated into the active tri-phosphate, with a half-life of 18 hours (13). The D-form of this analogue is more potent than the L-form (14, 15) and competes with dGTP for incorporation in the viral chain. The K_i is approximately three times lower than the K_m of the natural nucleoside (14).
Entecavir is a carbocyclic 2'-deoxyguanosine analogue (Fig 3C). In hepatoma cell lines, the active tri-phosphate concentration inside the hepatocyte accounts for 60-80% of all nucleotides (6). The tri-phosphate metabolite has a half-life of around 15 hours as measured in cell systems (6).
As shown in *in vitro* studies of the effect of entecavir and lamivudine on duck hepatitis B virus replication, priming is inhibited by entecavir and not by lamivudine (11). Both reverse transcription and (+) DNA strand synthesis are inhibited by both compounds, but lamivudine exhibits 15-150 and 5-25-fold less activity in inhibiting reverse transcription and (+) DNA strand synthesis, respectively, compared to entecavir (11).
Ganciclovir is an ethoxymethyl guanine with moderate *in vitro* activity against replicative intermediates of the duck hepatitis virus (Fig 3D) (16). One *in vitro* study on antiviral activity of ganciclovir and famciclovir indicates that famciclovir causes more pronounced suppression of viral replication than ganciclovir (14). One possible mechanism could be that famciclovir is more stable intracellularly than ganciclovir.
Adefovir dipivoxil (bis-POM-PMEA) is an acyclic nucleoside phosphonate analogue, which differs from the nucleoside analogues because this drug contains an incorporated phosphate (Fig 3E). After absorption, bis-POM is removed from the parental drug resulting in the actual antiviral agent adefovir. For activation of this drug, phosphorylation to the monophosphate, can be omitted (17) and only one additional step of phosphorylation is required for production of the active drug. The intra-cellular half-life of adefovir differs according to the cell-system (5-49 hours) (18). In addition, adefovir is believed to stimulate NK-cell activity and to induce endogenous interferon production, which may enhance viral eradication (19).

Covalently Closed Circular DNA (cccDNA)

The mechanism of conversion of the partially double stranded DNA virus to covalently closed circular DNA (cccDNA) after entry of the virus into the hepatocyte is not completely clarified at present. Early reports claim that this process is catalyzed by the viral polymerase (20, 21). Recent *in vitro* experiments illustrate that nucleoside analogues probably cannot inhibit this conversion to cccDNA (1, 2) and that the amount of cccDNA in woodchuck hepatocytes does decrease during ddC or lamivudine therapy, but only to the extent of

hepatocyte loss in these cell cultures. Another study performed in a recombinant baculovirus-HepG2 system (22) revealed that the accumulation of cccDNA was markedly inhibited if lamivudine was added to the system before infection. This may indicate that lamivudine does have some effect on the formation of cccDNA after entry of the virus into the hepatocyte but not on accumulation of cccDNA after infection has been established.
In studies on treatment of ducks infected with the hepatitis B virus with famciclovir and ganciclovir, cccDNA was not eradicated during 24 weeks of treatment. It is postulated that famciclovir and ganciclovir only affect the internal conversion pathway and not the conversion of partially double stranded relaxed virus to cccDNA after entry into the cell (15, 16, 23).
Although the results of these studies are not unequivocal, it is plausible to assume that the infection of uninfected cells cannot be blocked by nucleoside analogues since this step is mediated by cellular polymerases. Only re-entrance of the virus into the nucleus to replenish the pool of cccDNA may be inhibited by nucleoside analogues as a result of interference with reverse transcription. In clinical situations, this implies that, if therapy is administered long enough, the pool of cccDNA may eventually vanish. Studies of woodchucks indirectly showed that entecavir might have a direct effect on the cccDNA pool; induction therapy followed by maintenance therapy (one dose once a week) for 52 weeks caused undetectable markers of the hepatitis B virus in all treated animals. After withdrawal of the drug, five out of six woodchucks showed absence of the hepatitis B virus until 17 weeks after withdrawal of therapy (25). This may indirectly indicate that the cccDNA pool has been reduced drastically. The exact mechanism of entecavir on cccDNA still needs to be determined.
New studies on woodchucks treated with adefovir have also indicated that the cccDNA pool is very stable and cannot be decreased by interference of this adenine nucleoside analogue during 24 days of therapy. During longer therapy, adefovir may have more effect on cccDNA since this drug is also capable of inducing NK-cell activity and stimulating endogenous interferon production. These features may help to kill infected hepatocytes and diminish the pool of cccDNA (26).

Safety

The safety of nucleoside analogues should be evaluated in the light of *in vitro* data on cytotoxicity and the affinity of these analogues for cellular polymerases. The therapeutic index is an *in vitro* ratio, which expresses the concentration of the drug which kills 50% of the hepatocytes in relation to the concentration of the drug needed to inhibit 50% of the viral replication (CC_{50}/IC_{50}). If the K_i/K_m ratio is known for cellular polymerases, it provides information about the affinity of the analogue for cellular polymerases, possibly causing toxicity. Several cellular polymerases are involved in the replication of the cellular genome. Polymerase γ is responsible for replication of mitochondrial DNA. Polymerase α and δ are mediators of genome replication whereas polymerase β is involved in repair of the genome. Polymerase ε is most likely involved in both replication and repair. Insight into interference of the nucleoside analogues with these different cellular enzymes is essential for understanding and evaluating the potential toxicity.
As observed during therapy with fialuridine, a thymidine analogue, the close resemblance to natural nucleotides may cause serious clinical problems (27, 28). The therapeutic index of

fialuridine is satisfactory, as shown in *in vitro* studies (29). However, fialuridine exhibited an high affinity for polymerase γ, which incorporates the drug into mitochondrial DNA (30). This results in a completed but deviant DNA chain. Due to this aberrant DNA, oxidative phosphorylation can no longer take place, causing lactic acidosis, neuropathy, myopathy, pancreatitis and even death. Either one of the polymerases can incorporate fialuridine with a higher affinity than the natural nucleoside (dTTP) (30). Because polymerase γ has the lowest K_i of these polymerases, incorporation into this polymerase seems to cause the biggest problem. Even though polymerase γ, δ and ε exhibit exonuclease activity (31), incorporated fialuridine does not seem to alter the tri-dimensional structure in such a way that the disruption is recognized and removed by the exonuclease activity. Drugs like lamivudine, entecavir, famciclovir, ganciclovir and adefovir do not exhibit, or only to a lesser extent, these qualities (32-34).

Resistance

Five subsequent domains encompassing nucleotides 423 through 605 (genotype A) (A, B, C, D and E) are highly conserved in both the RNA-dependent HBV DNA polymerase and RNA polymerases (Fig 2). For the Human Immunodeficiency Virus (HIV), which contains a polymerase that is highly comparable to the hepatitis B polymerase, it is proposed that domains A, C and D are directly involved in nucleoside binding; domains B and E may be involved in positioning of the primer and the template (35). Structural changes in these domains in particular may be responsible for resistance to nucleoside analogues.

Table 1 Nucleotide changes inducing resistance to lamivudine and famciclovir in genotype A HBV.

Lamivudine	Polymerase domain	Famciclovir	Polymerase domain
I401S		V521L	B-domain
F514L	B-domain	P525L	B-domain
V521L	B-domain	L528M	B-domain
L528M	B-domain	L528V	B-domain
V528M	B-domain	T532S	B-domain
T532S			
A548V	C-domain		
M552I	C-domain		
M552V	C-domain		
M552S	C-domain		
V555I	C-domain		
S561T			
L577V			

Lamivudine and famciclovir have been extensively evaluated for the emergence of mutations in the polymerase gene *in vitro* and *in vivo* (Table 1). Prolonged lamivudine therapy is hampered by the emergence of mutations at the catalytic site of the polymerase gene, the so called YMDD motif in domain C. In this motif, the methionine at position 552 is replaced by valine or isoleucine; the former being most often associated with a mutation in the B-domain of the polymerase gene (substitution of a leucine for a methionine nucleoside at position 528) (36-45). This mutation causes a change in the tri-dimensional structure of the polymerase; subsequently lamivudine is less efficiently incorporated into the viral chain, because of reduced spatial fit in the polymerase structure. As a result, reverse transcription proceeds with natural nucleosides and a rebound of viral replication occurs. Other mutations in both the B-domain and the C-domain of the polymerase gene caused by lamivudine have been described (Table 1).
Famciclovir induces mutations in the B-domain. The mutation at position 528 (leucine replaced by either methionine or valine) is also known to occur during lamivudine therapy and may therefore cause cross-resistance (43-46).

A search for new antiviral therapies should focus on the different mechanisms of action of antiviral drugs to bypass the problem of resistance. Entecavir and adefovir have proven to have a continuing effect on lamivudine resistant strains *in vitro* (41, 47) and *in vivo* (48) (Table 2). Even with the structural change induced by lamivudine, the viral polymerase is still capable of incorporating other analogues into the catalytic site. Until now, follow-up data on treatment with entecavir, ganciclovir and adefovir have not shown resistance *in vivo*; however, these agents have only been administered for a short period. If entecavir and adefovir exhibit stronger viral suppression than lamivudine, as has been observed in *in vitro* studies (11, 49), combined with a different interaction of the analogue with the polymerase, it is reasonable to assume that the emergence of mutations may take longer to evolve.
The rate at which mutations emerge is influenced by several factors and may be lower if stronger viral suppression is achieved (50). Secondly, the reverse transcriptase does not have a proof-reading capacity to excise nucleoside analogue monophosphates which have been incorporated (51, 52). This makes the virus more prone to mutations.

Table 2 Fold increase in K_i of nucleoside analogues for lamivudine induced mutants compared to wildtype.

Nucleoside analogue	wildtype	M552I	M552V	L528M	L528M/M552V
Lamivudine[44]	1	8.0	19.6	2.6	25.2
Famciclovir[39]	1	1.1	3.1	3.1	2.5
Adefovir[44]	1	1.3	2.2	2.3	0.79

Clinical application

Data on results from *in vitro* studies and animal models lead the way to optimal application of new nucleoside analogues in chronic hepatitis B patients. Lamivudine has proven its worth by inducing strong inhibition of viral replication, reduction of liver inflammation and

improvement of liver histology (53-56). Recently it has been registered for the treatment of chronic hepatitis B. However, the emergence of mutations during long-term monotherapy prevents the drug from being used as a single agent to eradicate the virus from the liver (36-44).
Although promising in *in vitro* studies, famciclovir and ganciclovir, which have established their value as therapies for herpes viruses and the cytomegalovirus, respectively, have only limited *in vivo* effect against the hepatitis B virus (57-59). Monotherapy with these agents has therefore been abandoned. Possibly, they can still be used as a part of nucleoside analogue combination therapy (60). In contrast, adefovir dipivoxil and entecavir, which both have a good safety profile *in vitro*, have proven their *in vivo* efficacy in chronic hepatitis B patients also (61-63). They are being evaluated in phase III studies at the moment. In early studies, the use of adefovir dipivoxil in the dose range 60 to 120 mg once daily was limited by nefrotoxicity. Present studies are ongoing with dosages between 10 and 30 mg to avoid this side-effect (64).

Conclusions

Optimal antiviral therapy for the majority of chronically infected hepatitis B patients, resulting in loss of HBV DNA, HBeAg, and eventually HBsAg, cannot be obtained with nucleoside monotherapy. New nucleoside analogues enter the area of clinical application on a regular basis and a balance should be found between safety and optimal viral suppression. Combinations of nucleoside analogues with slightly different mechanisms of interference with the viral polymerase may prevent or at least delay the moment of the development of a mutant virus. This would make it possible to clear cccDNA from hepatocytes by natural mechanisms, such as hepatocyte division and possibly intrinsic immunity, to completely eliminate the virus.

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2

VIRAL DYNAMICS IN CHRONIC HEPATITIS B PATIENTS DURING LAMIVUDINE THERAPY

L.M.M. Wolters[1], B.E. Hansen[2], H.G.M. Niesters[3], S. Zeuzem[4], S.W. Schalm[1], R.A. de Man[1]

Department of [1]Gastroenterology & Hepatology, [2]Epidemiology & Biostatistics and [3]Virology, University Hospital Rotterdam, The Netherlands; [4]Department of Internal Medicine, JW Goethe-Universität, Frankfurt am Main, Germany.

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Summary

Rationale and aim Recently, we described a first order decay model for the description of a decrease in viral load during treatment with lamivudine for a chronic hepatitis B virus infection (HBV). However, more frequent sampling of viral load during the first month of treatment shows a bi-phasic viral decline. We therefore compared several mathematical models which are currently in use to describe the dynamics of various viruses and treatments.
Methods HBV DNA positive chronic hepatitis B patients were treated with lamivudine 150-600 mg daily for four weeks. During the first two days, blood samples were drawn every six hours, then daily during the first week and weekly during the following three weeks. HBV DNA was measured with the Digene Hybrid Capture II HBV DNA test and the sensitive Roche PCR assay, both calibrated on the Eurohep standard.
Results Our HBV DNA data are most accurately described if we use the bi-phasic model previously described by Neumann et al. while introducing all consecutive data of all patients simultaneously (mixed effects model). This results in an effectiveness of blocking of viral replication of 93%, a half-life of free virus of 17 hours and a half-life of infected hepatocytes of 7 days in patients treated with 150 mg of lamivudine.
Conclusion HBV dynamics during treatment with lamivudine can be explained by blocking of virion production with or without blocking of de-novo infection. The bi-phasic model as described by Neumann et al. in combination with frequent blood sampling, provides the most accurate fit and can be used to compare new nucleoside analogue profiles to lamivudine therapy.

Introduction

In patients who are chronically infected with the hepatitis B virus, response to therapy can be described in various ways. Most larger studies report on log viral decline during a specified time-interval (1-5). However, since the early studies were based on viral decline with insensitive assays, response to therapy most often resulted in description of the percentage of patients who became negative with this assay. The introduction of sensitive Polymerase Chain Reaction (PCR-) assays, has provided us with a tool to evaluate viral decline in more detail (6). By using these assays, it has been shown that patients who were considered responders in the early studies, still may have a considerable amount of virus in serum even after several months of therapy (7).
Mathematical description of viral decline during therapy is a second option to compare the response to therapy between individual patients and groups of patients. Models that describe the biological sequence of events which take place during antiviral therapy for chronic hepatitis B patients have been developed since 1995 (8, 9). In one of these models (8) we described a first order decay model of viral decline during lamivudine therapy; however, frequent sampling during the first month of therapy has revealed a bi-phasic viral decline. The most recent model is a model which was originally developed for the description of viral decline in chronic hepatitis C patients during alpha interferon therapy (10). Viral decline in chronic hepatitis B patients who are treated with adefovir dipivoxil has proven to fit well with this model (11).
In our study, we evaluated the three different published models on description of viral decline during treatment in chronic hepatitis B patients. Moreover, we compared two different statistical techniques to compare the results of the fit of the model which fitted the observed data most accurately. For comparison of mathematical models and statistical techniques, we used data of chronic hepatitis B patients who were treated with lamivudine.

Patients and methods

Patients
Twenty-one chronic hepatitis B patients with active viral replication (HBV DNA > 1.5×10^6 genome equivalents/ml (geq/ml)) were randomly assigned to receive either 150 mg (n=11) or 600 mg (n=10) of lamivudine daily for 4 weeks. After this initial treatment period all patients continued to receive 150 mg lamivudine daily. Randomisation was computer-generated and randomisation labels were kept in sealed non-opaque randomisation envelopes. Blood samples were drawn at screening, baseline and at t=6, 12, 18, 24, 30, 36, 42 and 48 hours. Subsequently, blood samples were obtained daily during the first week and at day 7, 10, 14, 21 and 28. Samples were snap frozen and stored. At each visit HBV DNA was assessed.

HBV DNA measurement
HBV DNA was quantified with a Digene Hybrid Capture tube liquid hybridisation assay (calibrated on the EUROHEP standard (12)). If HBV DNA became undetectable with this assay (limit of detection 1.5×10^6 geq/ml) during lamivudine treatment, it was reassessed with the quantitative PCR (Roche, Amplicor Diagnostics, Almere, The Netherlands calibrated on the EUROHEP standard; a lower limit of detection of 1000 geq/ml).

Models for viral dynamics

The viral dynamics for the two dosage groups were analysed by means of a single exponential model (Zeuzem et al (8)) and a bi-phasic model with a flat second phase (hereafter referred to as bi-phasic model 1) by Nowak et al. (9). Both models (8,9) described the viral decline in chronic hepatitis B patients treated with lamivudine. We compared these two models with a bi-phasic model defined by Neumann et al. (10) for hepatitis C (hereafter referred to as bi-phasic model 2) which was also used, with some modifications, for the treatment of chronic hepatitis B patients with adefovir by Tsiang et al. (11).

Both the exponential and the bi-phasic model 1 and 2 are based on three differential equations which describe the equilibrium between uninfected hepatocytes (T), infected hepatocytes (I) and free virus (V) (6).

$dT/dt = s - dT - (1-\eta)\beta VT$

$dI/dt = (1-\eta)\beta VT - \delta I$

$dV/dt = (1-\varepsilon)pI - cV$

s = production rate of uninfected cells

d = death rate of uninfected cells

β = infection rate of uninfected cells by the free virus

δ = death rate of productively infected cells

p = viral production rate from infected cells

c = clearance rate of the free virus

ε = effectiveness of lamivudine in blocking virion production from infected cells

η = effectiveness of lamivudine in blocking de novo infection of uninfected cells

A pure exponential decay model (8) is based on total inhibition of viral production by the antiviral agent ($\varepsilon=1$) and possibly total inhibition of infection of uninfected cells ($\eta=1$), resulting in the following decay function:

$V(t) = V_0 e^{-ct}$

For the bi-phasic model 1 (9) it was assumed that the number of infected cells (I) does not considerably decrease during early treatment and reaches a plateau level $(1-\varepsilon)V_0$ during the treatment of hepatitis B patients with lamivudine.

$V(t) = (1-\varepsilon)V_0 + \varepsilon V_0 e^{-ct}$

For the bi-phasic model 2 (10) it is assumed that:

1. the number of uninfected cells (T) does not considerably increase throughout the first 4 weeks of treatment.
2. the major antiviral effect of lamivudine is to partially block virion production from infected cells ($0<\varepsilon<1$) thus causing a rapid decline in the first few days with the slope depending on clearance of free virus (c).

This results in the following equation:

$V(t) = V_0\{A\exp[-\lambda_1 t] + (1-A)\exp[-\lambda_2 t]\}$

$A = (\varepsilon c - \lambda_2)/(\lambda_1 - \lambda_2)$

$\lambda_{1,2} = \frac{1}{2}\{(c+\delta) \pm [(c-\delta)^2 + 4(1-\varepsilon)(1-\eta)c\delta]^{1/2}\}$

Neumann et al. (10) assumed that $\eta=0$ (no block of de novo infection of uninfected cells), while Tsiang et al. (11) assumed that $\eta=1$ (complete block of infection of uninfected cells). We considered both assumptions as a possibility and we report a minimal and maximal estimate for δ which is the only variable significantly dependent on η.

Statistics

The three models were fitted with mixed modeling techniques using random effects. This allows for analysis of the entire data set with the possibility to estimate dose specific dynamics parameters and curves. The random effects (subject specific corrector factors) included for each parameter give the individual deviation for the dose-specific curves.

Previous analysis of dynamic models used the method of an individual fit per patient in contrast to our method analysing group effects using the entire data set. In order to compare these analysis methods, we applied individual and group fits using the bi-phasic model 2. Mixed modeling implies a group wise analysis while introducing random effects on all parameters and is most precise from a statistical point of view since all variables as well as all patient data are related and influenced by each other. Based on these data, group effects can be derived and compared.

For the exponential decay, PROC MIXED in SAS 6.12 was applied. Non-linear modeling was used to fit the bi-phasic model 1 and the bi-phasic model 2, executed in the NLINMIX macro in SAS 6.12.

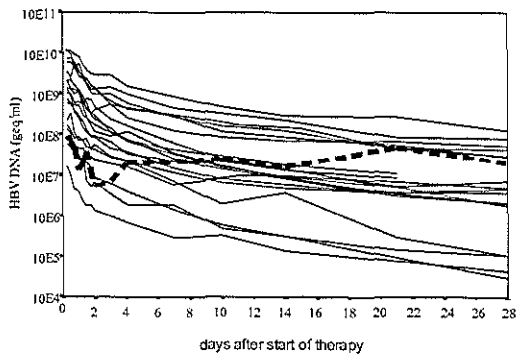
To quantitatively describe the accuracy of the several models, Akaikes Information Criteria (AIC) were assessed (13). The AIC represents the relation between the 2log likelihood and the number of degrees of freedom. The AIC closest to zero indicates the smallest residuals from the observed HBV DNA data, resulting in the best fit.

Results

All the patients showed a very similar bi-phasic viral decline pattern, except for patient 3, who had a rebound in viral replication after an initial decline (Fig 1).

All treated patients were analysed and corrections of dose were accounted for. To compare different modeling techniques, the following results and the results in both tables describe patients treated with 150 mg of lamivudine.

Fig 1 Viral decline of individual patients. All patients, except patient 3 (in dashed line), show a consistent pattern of decline. To compare the three different models, patient 3 is included in the analysis (Table 1). However, to compare the individual fitting with the mixed effects approach of the bi-phasic model 2, this patient is excluded (Table 2). Since this patient shows a rebound in viral load, the death rate of infected cells (δ) which partially determines the viral decline during the second phase cannot be calculated using individual fitting techniques.



Comparison of the fit of the different models

Both the visual assessment (Fig 2) as well as the AIC of the exponential model (-456) and the bi-phasic model I (-359) show that these are not accurate fits of our patient data. In contrast, the AIC of the bi-phasic model 2 (-220) as well as the observed fit to the data-points shows that this model does represent the best fit for these patient data compared to the other models (Fig 2 and Table1 I).

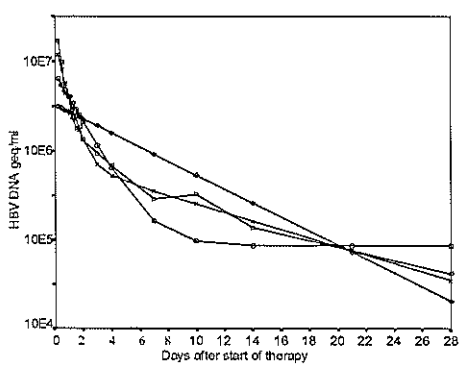
The exponential model yields in a viral half life of 108 hours (Table 1). For the bi-phasic model 1, the death rate of free virus and the derived viral half life is 29 hours. This viral half life reflects the initial first phase of rapid viral decline since the second phase is expressed by a constant (ρ) which does not add to further viral decline. In the bi-phasic model 2, effectiveness in blocking viral production (ε), half-life of free virus, and half life of infected cells are 0.915 (91.5%), 15 hours and 186 hours respectively (Table 1).

Table 1 Fitting of HBV decline during lamivudine treatment with the 3 models based on the group mixed effect approach (including patient 3).

	150 mg/day mean fit (s.e.)	overall fit (AIC)
Exponential decay:		
V_0e^{-ct}		
T½ (ln2/c)	108 hrs {CI 89-139}	-456
Nowak:		
$(1-\varepsilon)V_0+\varepsilon V_0e^{-ct}$		
ε	0.973 (0.008)	-359
T½ (ln2/c)	29 hrs {CI 25-35}	
Neumann:		
$V_0(Ae^{-\lambda_1 t}+(1-A)e^{-\lambda_2 t})$		
ε	0.915 (0.016)	-220
T½ (ln2/c)	15 hrs {CI 12-19}	
T½ (ln2/δ) (if η=0)	186 hrs (≈ 8 days) {CI 140-277}	

V_0 = initial viral load
c = clearance rate of free virus
δ = death rate of productively infected cells
ε = effectiveness of lamivudine in blocking virion production
CI = 95% Confidence Interval
s.e. = standard error
AIC = Akaikes Information Criteria
T½ = half-life

Fig 2 HBV DNA of individual 9 fitted by the exponential model (diamonds), the bi-phasic model 1 (circles) and the bi-phasic model 2 (crosses). The bi-phasic model 2 provides the best fit of the observed data (squares).



Comparison of individual and group-wise fitting

If patients are fitted individually, a variable delay in viral decline can be introduced. Mathematically a variable delay could also be applied for the mixed effects approach, but this

results in too many variables in relation to the amount of patient data. For the mixed effects approach, we therefore introduced a fixed delay in viral decline (six hours): the first HBV DNA measurement after start of therapy was at six hours. Patient 3 is the only patient who does not show a bi-phasic viral decline; the initial fast decrease of viral replication is followed by a rebound in viral load. Therefore, the death rate of infected hepatocytes (δ) which partially determines the viral decline during the second phase can not be calculated for this individual patient. To compare the individual and group-wise fitting, patient 3 is excluded.
Individual modeling for the bi-phasic model 2 which is dependent on c, δ, ε and η, results in an effectiveness in blocking viral production of 0.922 (92.2%), half-life of free virus of 16 hours and a half-life of infected cells of 178 hours. If a group-wise fit of the bi-phasic model 2 is applied, the effectiveness in blocking viral production (ε), half-life of free virus, and half life of infected cells are 0.928 (92.8%), 17 hours and 177 hours respectively (Table 2). If patient 3 is included into the mixed effects analysis, half-life during the second phase is slightly elongated (186 hours versus 177 hours; Table 1 and 2).

Table 2 Parameter estimates based on the bi-phasic model 2 with individual non-linear fitting and mixed effect group fitting (excluding patient 3).

	150 mg/day mean fit
Bi-phasic model 2 (individual fit):	
$V_0(Ae^{-\lambda_1 t}+(1-A)e^{-\lambda_2 t})$	
ln (V_0)	20.22 (s.d. 2.23)
c (with a variable delay)	1.238 (s.d. 0.590)
δ (if η=0)	0.099 (s.d. 0.062)
δ (if η=1)	0.092 (s.d. 0.049)
ε (with a variable delay)	0.922 (s.d. 0.032)
T½ (ln2/c)	#16 hrs(range 9-42)
T½ (ln2/δ) (if η=0)	#178 hrs (≈7 days)(range 84-768)
Bi-phasic model 2 (group fit):	
$V_0(Ae^{-\lambda_1 t}+(1-A)e^{-\lambda_2 t})$	
ln (V_0)	20.13 (s.e. 0.65)
c (with fixed delay of 6 hours)	0.979 (s.e. 0.11)
δ (if η=0)	0.094 (s.e. 0.014)
δ (if η=1)	0.086 (s.e. 0.014)
ε (with fixed delay of 6 hours)	0.928 (s.e. 0.015)
T½ (ln2/c)	17 hrs (CI 14-22)
T½ (ln2/δ) (if η=0)	177 hrs (≈7 days)(CI 137-250)

V_0	= initial viral load	CI	= 95% Confidence Interval
c	= clearance rate of free virus	s.e.	= standard error
δ	= death rate of productively infected cells	s.d.	= standard deviation
η	= effectiveness of lamivudine in blocking de novo infection of uninfected cells	#	= median
ε	= effectiveness of lamivudine in blocking virion production		
T½	= half-life		

Discussion

Modeling gives us a tool for evaluation of the effect of an antiviral therapy, by means of parameters of effectiveness and both viral half-life and half-life of infected cells. This is in contrast with rough data on viral decline during therapy which are merely a surrogate marker of the effectiveness of the drug. In addition, modeling probably can be used to predict response to therapy in individual patients and to compare different nucleoside analogues or nucleoside analogue combinations.

The aim of this study was to examine which model describes the HBV DNA data most accurately and to explore the best available statistical technique (mixed modeling techniques using random effects versus individual fits) to describe the decline of the hepatitis B virus during lamivudine therapy. The mixed modeling technique shows that the bi-phasic model 2 most accurately reflects the observed data (AIC closest to zero and observed by visual assessment) compared to the exponential model and the bi-phasic model 1.

Our data are in agreement with viral decline during adefovir therapy as described by Tsiang et al. (11). The model which has been used in this publication is comparable to our bi-phasic model 2. However, frequent blood withdrawal seems to be essential to accurately describe the biological phenomena. Effectiveness (ε) of adefovir in this study was 0.993. The effectiveness of lamivudine as calculated by mixed modeling in our study is lower: 0.928. In contrast, the mean viral half-life during the first phase in the adefovir study is 1.1 day and the half-life of infected cells during the second phase is 18.2 days. This is considerably longer than 17 hours for the first phase and 177 hours for the second phase in our study. We calculated a duration of the initial phase of 2.7 days, such that the change from the first to the second phase occurs early in the first week as can be observed from our data. Tsiang et al. conducted the first HBV DNA measurement after one week of therapy, which may explain these discrepant findings. Although the exact frequency of observations that is required for optimal modeling needs to be determined yet, the first days of therapy are important and need to be documented adequately. From our data, we would recommend minimal blood withdrawal at day 0, day 2, 4 and 7.

The exponential model provides the least precise fit of the three applied models. *In vitro* assays show that nucleoside analogues, lamivudine in particular, probably cannot prevent infection of uninfected cells (14,15). The conversion of the double stranded viral genome to cccDNA is mediated by a cellular polymerase for which lamivudine has much less affinity than the natural cytosine (16,17). Also, complete inhibition of viral replication would very soon lead to disappearance of the virus from the blood and eventually from the liver. In contrast, long-term follow-up of patients during lamivudine therapy shows that a considerable proportion of these patients retain a moderate viral activity, with detectable HBV DNA by insensitive assays after several months of treatment (6).

The bi-phasic model 1 takes into consideration the change in half-life of the virus during treatment. However, the fit of our patient data shows that the initial fast viral decline is not followed by a complete steady state of virus production and degradation, which is expressed in this model. Although viral decline is slower during the second phase, a continuing decrease of viral load in serum can still be observed.

The assumptions made in the bi-phasic model 2 also hold true for our patient group. The major effect of lamivudine is near complete blocking of replication of the virus in hepatocytes, causing a reduction of release of free virus into the peripheral blood. However, lamivudine does not influence the degradation of free virus since this drug has no immunomodulating activity nor does lamivudine have a toxic effect on infected hepatocytes. We can not rule out a possible

effect of lamivudine on blocking infection (η varying between 0% and 100%), but clearly the viral kinetic data can be fit without assuming such an effect.

According to our data, application of the mixed modeling technique for the bi-phasic model 2 provides an optimal fit for description of viral decline in chronic hepatitis B patients during lamivudine therapy. Lamivudine blocks production of virus (ε), which we can measure. In addition, lamivudine may also have some direct effect on the infection of uninfected cells. However, this cannot be shown with the bi-phasic model 2, since the second phase of viral decline, during which lamivudine could interfere with infection of uninfected cells, is dependent on too many variables.

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3

THE INFLUENCE OF BASELINE CHARACTERISTICS ON VIRAL DYNAMIC PARAMETERS IN CHRONIC HEPATITIS B PATIENTS TREATED WITH LAMIVUDINE

L.M.M. Wolters[1], B.E. Hansen[2], H.G.M. Niesters[3], R.S. Levi-Drummer [4], A.U. Neumann[4], S.W. Schalm[1], R.A. de Man[1]

Department of [1]Gastroenterology & Hepatology, [2]Epidemiology & Biostatistics and [3]Virology, University Hospital Rotterdam, The Netherlands; [4]Faculty of Life Sciences, Bar-Ilan University, Israel.

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Summary

Viral decline during lamivudine therapy in chronic hepatitis B patients is bi-phasic. We studied the influence of lamivudine dose and baseline characteristics on parameters obtained from a mathematical model.

Methods Chronic hepatitis B patients were randomised to receive 150 mg (group 1; n=11) or 600 mg (group 2; n=10) lamivudine daily for four weeks. HBV DNA was measured frequently with the Digene Hybrid Capture II test and the Roche PCR assay.

Results Description of viral decline in our closely monitored patients by means of the mixed effects approach with both the bi-phasic model and a piecewise linear regression model resulted in a good fit. Baseline ALT was significantly related to the slope of the second phase of viral decline. Previous lamivudine treated patients showed a significant slower first phase than patients naïve to lamivudine treatment.

Conclusion The initial observed difference in viral decline between 150 mg and 600 mg of lamivudine disappeared when baseline ALT was taken into account. This strengthens the hypothesis that the level of intrinsic activity is related to the turnover of infected hepatocytes. Moreover, reintroduction of lamivudine in previously lamivudine treated patients should be considered carefully.

Introduction

Lamivudine is capable of inhibiting hepatitis B viral replication in the majority of patients if doses over 100 mg daily are applied (1, 2). Previous studies on the effect of lamivudine were based on description of the percentage of patients with a viral decline below the limit of the insensitive assay used (1- 4). However, recent studies, which were based on mathematical description of viral decline, are able to reveal more detailed differences between patients (5-7). A bi-phasic model which was originally evaluated for hepatitis C viral decline during alpha interferon therapy (7) has proven to describe adequately viral decline in chronic hepatitis B patients treated with nucleoside analogues also (8,9). In contrast to the previous described data in adefovir therapy (8), we tried to increase the accuracy of the estimates by increasing the frequency of first week sampling for HBV DNA levels.
In analogy to the response of chronic hepatitis B patients to alpha interferon therapy, which is largely dependent on baseline factors (10-12), lamivudine treated patients also show a higher chance of HBeAg seroconversion if intrinsic activity against the HBV, as reflected by elevated serum transaminases, is present at baseline (11,13). Repeated alpha interferon therapy in previous non-responders induces a similar percentage of HBeAg seroconversion compared to treatment of alpha interferon naïve patients (14). However, previous failure to lamivudine therapy could alter the response of repeated therapy due to altered characteristics of the virus or selection of patients. In order to evaluate the effect of baseline factors on response to therapy, we evaluated high and low dose lamivudine treated patients.

Patients and methods

Patients
Eligible patients included men and women between 18 and 70 years of age who had a biopsy-proven chronic hepatitis B virus infection and who were positive for HBsAg. HBV DNA had to be $>1.5x10^6$ genome equivalents/ml (geq/ml). Patients were excluded if they had detectable antibodies against the hepatitis C or D virus or if they were co-infected with the Human Immunodeficiency Virus (HIV). Patients with decompensated liver disease, liver diseases with other aetiologies or other serious concomitant medical illnesses were excluded as well. The Medical Ethics Committee of the University Hospital Rotterdam approved the study. All patients had to give written informed consent.

Twenty-one patients were randomly assigned to receive either 150 mg or 600 mg of lamivudine daily for 4 weeks. After this initial treatment period all patients continued to receive 150 mg lamivudine daily. Randomisation was computer-generated and randomisation labels were kept in sealed non-opaque randomisation envelopes. All patients were screened one week before start of treatment for eligibility. Patients were admitted to the hospital for the first two days (day 0-2). Blood samples for HBV DNA measurement were drawn at screening, baseline and at t=6, 12, 18, 24, 30, 36, 42 and 48 hours, daily during the first week and at day 7, 10, 14, 21 and 28.

Virology
HBV DNA was quantified with a Digene Hybrid Capture tube liquid hybridisation assay (calibrated on the EUROHEP standard (15)). If HBV DNA became undetectable with this

assay (limit of detection $1.5x10^6$ geq/ml) during lamivudine treatment, it was reassessed with the quantitative PCR (Roche, Amplicor Diagnostics, Almere, The Netherlands calibrated on the EUROHEP standard with a lower limit of detection of 1000 geq/ml). HBV polymerase mutant analysis was performed with the INNO-LiPA DR-strip (Innogenetics, Gent, Belgium) (16).

Description of viral decline

The viral dynamics is described by an exponential bi-phasic model as previously described by Neumann et al. (7). In short, viral decline in the bi-phasic model is described by the following equation:

$V(t)= V_0\{Aexp[-\lambda_1 t]+(1-A)exp[-\lambda_2 t]\}$

where

λ_1 = slope of the first phase of viral decline
λ_2 = slope of the second phase of viral decline
A = $(\varepsilon c-\lambda_2)/(\lambda_1-\lambda_2)$
$\lambda_{1,2}$ = $\frac{1}{2}\{(c+\delta)\pm[(c-\delta)^2+4(1-\varepsilon)(1-\eta)c\delta]^{1/2}\}$
V_0 = initial viral load
T = time
δ = death rate of productively infected cells
c = clearance rate of the free virus
ε = effectiveness of lamivudine in blocking virion production from infected cells
η = effectiveness of lamivudine in blocking de novo infection of susceptible cells

The second slope is dependent on δ only if lamivudine is assumed to be capable to completely block the novo infection of uninfected hepatocytes (i.e. if $\eta = 1$, than $\lambda_2 = \delta$). However, if lamivudine does only in part or not block infection of uninfected hepatocytes at all, the second slope is expressed as λ_2 and dependent on c, δ, ε and η.

A piecewise linear model was also fitted based on the following equation:

$\log(V(t))= a+b*t+c_2*(t-t_b)*(t>t_b)$

$\log(V(t))= a+b*t$ if $t \leq t_b$
$\log(V(t))= (a-c_2*t_b)+(b+c_2)*t$ if $t > t_b$

where

V_0 = initial viral load
a = log V (t=0)
b = slope of the first phase of viral decline
$b+c_2$ = slope of the second phase of viral decline
t = time
t_b = break point

Statistics

The entire data set was analysed with mixed modeling techniques using random effects to estimate dose specific dynamic parameters and curves. The random effects (subject specific corrector factors) included for each parameter, give the individual deviation for the dose-specific curves. The data consist of 16 measurements per subject (n=21) (16x21=336 measurements). The mixed-effects model uses 4 fixed parameters (or 2x4 fixed parameters when extending the model with dose, see below) and 10+1 random parameters, that is ~5% of the data.

In contrast, the separate fit of the bi-exponential or the piecewise linear model to each of the 21 subjects uses 21x4 = 84 parameters. Moreover a separate fit does not take into account the obvious similarities among the individual curves. This is useful when one is interested in modeling the behavior of a particular, fixed set of individuals, but it is not adequate when the observed individuals are to be treated as a sample from a population of similar individuals, which constitutes the majority of applications involving grouped data. In our case, the interest is in estimating the average behavior of an individual in the population and the variability among and within individuals, which is precisely what mixed-effects models are designed to do (17).

For both models, non-linear mixed modeling was used, applying the NLINMIX macro in SAS 6.12. Akaikes Information Criteria (AIC) (18) were used to describe the accuracy of the models in relation to the observed patient data. To investigate a possible effect of baseline factors (pre-treatment ALT-values ≤2x ULN (Upper Limit of Normal) versus >2xULN, previous lamivudine treatment, Body Mass Index (BMI) and race) on the viral decline, covariates were included to the piecewise linear model. Each fixed parameter, for example λ_2 in the exponential bi-phasic model, is written as a sum of two parameters: one to explain the level of this parameter, $\lambda_{2,level}$, and one to explain the difference between the dose=150 and dose =600, $\lambda_{2,600}$, i.e.

$$\lambda_2 = \begin{cases} \lambda_{2,level} & \text{, if dose=150} \\ \lambda_{2,level} + \lambda_{2,600} & \text{, if dose=600} \end{cases}$$

Likewise, the model is extended with baseline factors simply adding an extra parameter to explain the difference, for example between high ALT and low ALT:

$$\lambda_2 = \begin{cases} \lambda_{2,level} & \text{, if dose=150 and ALT low} \\ \lambda_{2,level} + \lambda_{2,ALThigh} & \text{, if dose=150 and ALT high} \\ \lambda_{2,level} + \lambda_{2,600} & \text{, if dose=600 and ALT low} \\ \lambda_{2,level} + \lambda_{2,600} + \lambda_{2,ALThigh} & \text{, if dose=600 and ALT high} \end{cases}$$

Theoretically, the baseline variables could be incorporated into the exponential bi-phasic model, but with these extra variables, convergence could not be reached because of the complexity of the random-effect model and the number of variables. Instead, point estimates of the exponential bi-phasic model, derived from the non-linear fit per patient using ProcNLIN in SAS, were compared between groups with the non-parametric Mann Whitney test. Significant difference was achieved if $p<0.05$.

Patient 3 was the only patient with a rebound in viral replication during the second phase. For this individual patient, the exponential bi-phasic parameter of the second phase can not be interpreted. On the other hand, the course of viral decline represents one of the possible responses of wild-type virus to lamivudine. Therefore two sets of analyses were performed, one including patient 3 and one excluding patient 3.

Results

Twenty-one patients were analysed on a per protocol basis. Eleven patients received 150 mg lamivudine once daily for four weeks (group 1) and ten patients received 600 mg of lamivudine once daily for four weeks (group 2). Patients in the two groups were comparable with regard to age, sex, race and baseline viral load. Baseline serum transaminase levels were not significantly different between the two groups, although a wider range in the higher dose group was observed. Two patients in group 1 and 1 patient in group 2 were negative for HBeAg at start of therapy; all other patients had detectable HBeAg at start of therapy. The majority of patients were treatment naïve (Table 1). In patients who were treated with lamivudine previously, the drug was withdrawn at least 10 months before start of this study (median: 18 months, range 10-44 months). None of these patients had detectable YMDD mutant virus in blood at start of therapy. HBV DNA and Body Mass Index (weight divided by the square of the length in meters) at baseline were not significantly different between the two treatment groups (Table 1).

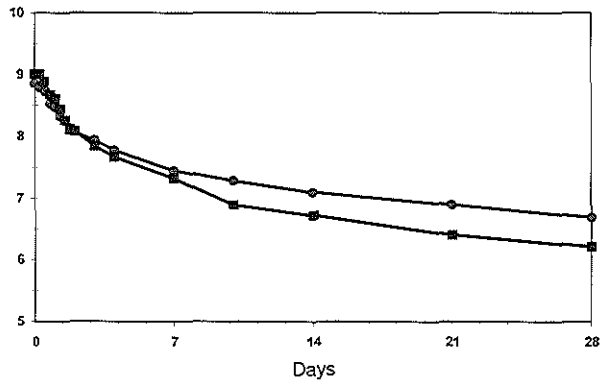
Table 1 Patient and pre-treatment characteristics

Treatment group	150 mg lamivudine	600 mg lamivudine
Male/female	9/2	9/1
Age (median, range)	27 years (18-51)	32 years (18-51)
Race		
Caucasian	2	5
Asian	5	2
Other	4	3
Previous therapy		
Lamivudine	1	3
Alpha interferon	2	2
Lamivudine/alpha interferon	2	1
None	6	4
Body Mass Index (median,range)	24.1 kg/m^2 (18.3-30.7)	22.4 kg/m^2 (19.4-30.0)
Cirrhosis	1	0
ALT (IU/l) (median,range)	47 (19-113)	90 (18-645)
HBV DNA (geq/ml; median, range)	$7.9x10^8$ ($2.8x10^7$-$1.3x10^{10}$)	$1.5x10^9$ ($8.5x10^7$-$7.6x10^9$)

Upper Limit of Normal is 30 IU/l.

Patient 3, who had a rebound after an initial viral decline had not received lamivudine previously. Results obtained from the analyses with and without patient 3 were similar. Data without patient 3 are not shown. The mean viral decline in group 1 and 2 is shown in Figure 1.

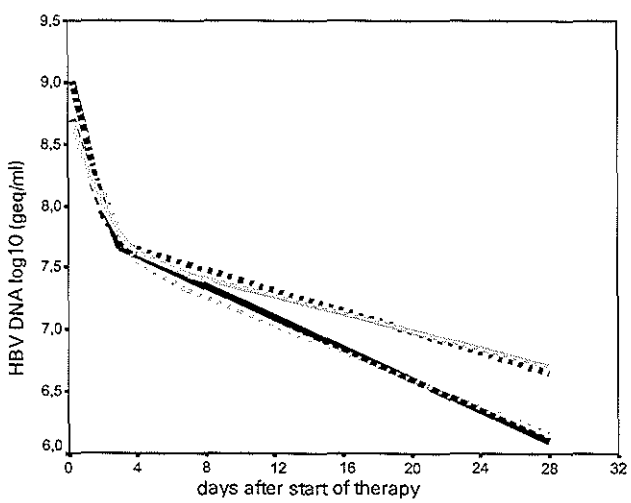
Fig 1 Mean HBV DNA decline on a log scale in group 1 (circles) and group 2 (squares)



Both the exponential bi-phasic model as well as the piecewise linear regression model fitted the observed HBV DNA data equally well, as observed by visual assessment (Figure 2) and expressed by a comparable AIC (-220 versus -243) (Table 2).

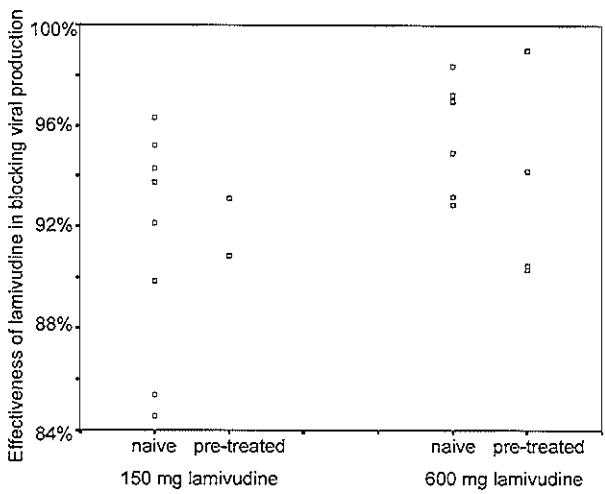
Fig 2 Representation of viral decline by means of a statistical fit of the two dose groups with the bi-phasic model and the piecewise linear model. Both fits practically overlap one another.

Straight black line: 600 mg, piecewise linear fit
Dotted grey line: 600 mg, bi-phasic fit
Dotted black line: 150 mg, piecewise linear fit
Straight grey line: 150 mg, bi-phasic fit



Comparison of the dynamical parameters between the two doses
The group-wise fit as well as the individual fit indicated a greater effectiveness in blocking viral production (ε) (Table 2 and Figure 3) and a shorter half-life of infected cells if a higher dose is applied (0.96 versus 0.92; p=0.028 and 125 hours versus 186 hours; p=0.030 respectively). Piecewise linear modeling showed similar results with a first phase of viral decline of 0.44 log/day for group 1 and 0.56 log/day for group 2 (p=0.064). This resembles a half-life of free virus of 13 hours in both treatment groups. Viral decline during the second phase was 0.041 log/day in group 1 and 0.063 log/day in group 2; i.e. a half life of infected hepatocytes of 174 hours in group 1 and 116 hours in group 2 (p=0.035). The break point of viral decline was calculated to be at 2.26 days in group 1 and 2.34 days in group 2 which was not significantly different.

Fig 3 Effectiveness of lamivudine in blocking viral production in relation to dose and previous lamivudine therapy.



Comparison of the dynamical parameters in relation to baseline factors
Baseline ALT was significantly related to the slope of the second phase: a higher ALT showed a faster decline (0.139 versus 0.095, p=0.03, including patient 3 and 0.155 versus 0.098, p=0.001, excluding patient 3). To support these findings, comparison of the point estimate of the per patient separate non-linear fit of the exponential bi-phasic model showed that λ_2 and δ were significantly higher for patients with ALT>2xULN (Mann-Whitney test; p=0.04, including patient 3 and p=0.03, excluding patient 3). However, if extending the model with dose, the baseline ALT was no longer significantly related to the slope of the second phase (p=0.18). Moreover, the difference between the two dose groups after correcting for ALT became less pronounced; a difference of 0.039 (p=0.11) versus a difference of 0.144-0.095=0.049 (p=0.035)(Table 2, i.e. including patient 3). This implies a possible correlation between the effect of dose and the baseline ALT.

Patients who were previously treated with lamivudine showed a trend to a slower initial viral decline compared to lamivudine-naïve patients (0.942 versus 1.257, p=0.06). Applying the Mann-Whitney test of the point estimate for c gave similar results, p=0.04 (including patient 3). After extending the model with dose, this difference even became significant (p=0.0001).
Body Mass Index and race were not related to any of the parameters calculated. Differences between sexes were not evaluated because only 3/21 patients were women.

Table 2 Parameter estimates based on the bi-phasic model and the piecewise linear regression model with mixed effect group fitting; population average with the range of individual patients.

	150 mg/day mean fit	600 mg/day mean fit	AIC	p-value (F-test)
Bi-phasic model:				
$V_0(Ae^{-\lambda_1 t}+(1-A)e^{-\lambda_2 t})$				
ln (V_0)	20.00 (range 16.26-23.18)	20.69 (range 17.77-22.87)	-220	0.438
c (with fixed delay of 6 hours)	1.13 (range 0.77-1.76)	1.34 (range 0.92-1.85)		0.257
δ (if η=1)	0.089 (range –0.03-0.19)	0.133 (range 0.08-0.21)		0.030
λ_2 (if η=0)	0.081 (range –0.03-0.18)	0.127 (range 0.08-0.19)		0.030
ε (with fixed delay of 6 hours)	92% (range 84-97)	96% (range 92-99.8)		0.028
half-life of free virus	15 hrs (range 9-22)	12 hrs (range 9-18)		0.257
half-life of infected hepatocytes	186 hrs (≈8 days)(range –516-331)	125 hrs (≈5 days)(range 79-210)		0.030
Piecewise linear regression model:				
$\log(V(t))=a+b*t+c_2*(t-t_b)*(t>t_b)$				
ln (V_0)	20.04 (range16.26-23.22)	20.75 (range 17.85-22.88)	-243	0.429
b	1.01 (range 0.70-1.32)	1.31 (range 0.82-1.81)		0.064
$b+c_2$	0.095 (range -0.04-0.20)	0.144 (range 0.08-0.20)		0.035
T_b (days)	2.26 (range1.53-2.71)	2.34 (range 1.99-2.58)		0.644
half-life of free virus	13 hrs (range13-24)	13 hrs (range 9-20)		0.064
half-life of infected hepatocytes	174 hrs (≈7 days) (range –275-430)	116 hrs (≈ 5 days) (range 82-205)		0.035

V_0 = initial viral load
c = clearance rate of free virus
δ = death rate of productively infected cells
ε = effectiveness of lamivudine in blocking virion production
η = effectiveness of lamivudine in blocking de novo infection of uninfected cells
a = log V (t=0)
b = slope of first phase
$b+c_2$ = slope of second phase
t_b = breaking point

Discussion

Viral decline during nucleoside analogue therapy for HBV consists of an initial phase of elimination of free virus and a second phase of the death rate of infected cells. Since infectious particles are also lost during cell division, δ probably reflects both cell death and division. Both the bi-phasic model and the piecewise linear model provide similar parameters, which describe the several biological processes that take place during the first weeks of antiviral therapy.

Modeling of the HBV DNA data resulted in a significantly steeper slope of viral decline during the second phase (δ or lambda 2 in the bi-phasic model and b+c in the piecewise linear model) for the higher dose group. Lambda 2 is a variable dependent on δ, η, ε and to a lesser extent c, which indicates that several factors might be responsible for the difference between the two doses. Biologically, δ can not be influenced by lamivudine because this drug has no hepatotoxic properties. However, if baseline ALT was accounted for, the initial difference in viral decline between the dose groups during the second phase seemed to disappear. This could indicate that δ, more than c, η and ε, was responsible for the faster viral decline in the second phase in group 2. Previous studies have shown that HBeAg seroconversion occurs significantly more often in those patients with elevated baseline ALT (13) compared to immunotolerant patients with normal ALT levels. This indicates that higher turnover of infected hepatocytes is followed by a faster occurrence of HBeAg seroconversion. However, it needs to be evaluated yet whether HBeAg seroconversion during lamivudine therapy is durable after withdrawal of therapy (19,20) and whether patients with higher baseline ALT are capable of developing a more durable HBeAg seroconversion. Based on our present knowledge, the primary action of lamivudine is inhibition of viral replication (21). One study reports on restoration of HBV-specific T-cells during lamivudine therapy in patients with hyporesponsive T-cells during active viral replication (22). However, this effect seemed to be induced through suppression of viral replication and not by lamivudine itself.

The definition of non-response to lamivudine is not unequivocal. Both continuing active viral replication after several months of therapy as well as breakthrough of variant HBV may be considered as a failing response. However, these responses to lamivudine are based on different mechanisms. Patients who have shown continuing active viral replication during previous lamivudine therapy without the development of variant virus may be less capable of optimal absorption, uptake in hepatocytes or phosphorylation in the hepatocyte (23). These are all host specific factors, which will probably be similar if lamivudine is applied again. Secondly, patients who have developed lamivudine resistance and who are withdrawn from therapy show a gradual return of wild-type virus. It has been described that lamivudine resistant virus was still detectable 4 months after withdrawal of therapy (24). Due to the limitations in the detection level of the tests used, absence of variant virus does not necessarily mean that it is not present in the viral population. With the INNO-LiPA DR-PCR-based assay, as few as 10^3 copies of mutant virus per ml and 4-8% of mutant virus in a population of wildtype virus can be detected (25). This could mean that our previously lamivudine treated patients indeed were still infected with mutant virus even though it cannot be detected. If variant virus was still present, reintroduction of lamivudine could cause renewed replication of the variant virus resulting in decreased decline of viral replication within the first few weeks of therapy. Lastly, selection of patients could also be an important factor for induction of the difference between previously lamivudine treated patients and lamivudine naïve patients. Further studies are needed to explore these findings.

Body Mass Index and race were not related to any of the parameters that were calculated in the model. Lamivudine is a hydrophilic compound that is not stored in fat after absorption (26).

Therefore, it seems reasonable to assume that both patients with a low and high body mass index respond similarly to lamivudine therapy. Race did not influence the response to therapy but is probably partially related to immune tolerant or active status as reflected by serum transaminases.
In our study with a high frequency of sampling during the first week of therapy the different response to different doses of lamivudine can be described in detail by both the bi-phasic model and the piecewise linear model. Higher intrinsic activity against HBV is related to a higher turnover of infected cells regardless of the dose of lamivudine. Previous non-response to lamivudine therapy seemed to have a negative effect on re-introduction of lamivudine. Therefore, re-institution of lamivudine should probably only occur if the clinical situation requires an intervention, e.g. in the case of flares of serum transaminases caused by HBV.

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4

LAMIVUDINE PLASMA LEVELS IN CHRONIC HEPATITIS B PATIENTS

L.M.M. Wolters[1], C.J.C. Geerlings[2], L. van Dijk[2], H.G.M. Niesters[3], A.G. Vulto[2], R.A. de Man[1]

Department of [1]Hepatology & Gastroenterology, [2]Pharmacy and [3]Virology, University Hospital Rotterdam, The Netherlands

Submitted

Summary

Lamivudine has recently been registered for the treatment of chronic hepatitis B patients. The main therapeutic outcome in the studies on which the registration was based, was a drop of HBV DNA below the level of detection of the insensitive Abbott assay. However, as reported previously by us, with the use of sensitive assays, individual differences in virologic response to lamivudine can be detected.

To analyse the chain of events after oral intake of lamivudine until the conversion to the active tri-phosphate, we modified and validated a High Pressure Liquid Chromatography (HPLC) method to evaluate lamivudine plasma levels. Lamivudine levels in chronic hepatitis B patients who participated in a study on the efficacy of lamivudine were comparable to our reference curve which was derived from 8 chronic hepatitis B patients. However, lamivudine exerts its action as the active tri-phosphate inside the hepatocyte after extensive handling. Therefore, additional steps in the pharmacokinetic process should be evaluated to explore the actual mechanisms that are responsible for the suboptimal response to lamivudine in a number of patients.

Introduction

Lamivudine, the negative enantiomer of 2'-3' deoxy 3' thiacytidine, is a nucleoside analogue which has recently been registered for the treatment of chronically infected hepatitis B patients. In large phase III studies the favourable effect of this drug was shown on suppression of HBV DNA -a parameter expressing active viral replication- that is often followed by a decline of transaminases and improvement of liver histology (1-3). The conclusions in these studies were based on the percentage of patients with a viral decline below the lower limit (approximately 10^7 genome equivalents/ml (geq/ml)) of the insensitive liquid hybridisation assays (Abbott Genostics, Abbott Laboratories, Abbott Park, IL). HBV DNA became undetectable in around 80% of patients as measured with this test after 6 months of therapy (1). However, if we look more carefully applying more sensitive assays, individual differences in response to lamivudine become apparent (4, 5). Whereas some patients show a rapid decline to levels even below the threshold of the qualitative PCR assay (Roche Monitor, lower limit of detection 400 geq/ml), in others, the hepatitis B virus (HBV) continues to replicate actively even after 6 months of therapy. We previously reported on a cohort of long-term lamivudine treated chronic hepatitis B patients in Rotterdam (4). In 19 patients with still detectable HBV DNA by insensitive assays (Digene, liquid hybridisation assay, lower limit of detection $1{,}5x10^6$ geq/ml) after six months of therapy, only three patients had a mutant virus which could explain this continuing active viral replication. Thus, ongoing active replication of the HBV must be based on some other phenomenon in the majority of patients. Lamivudine is subject to several transport and activation steps from oral intake until incorporation into the pregenomic viral chain. Our hypothesis was that unfavourable transport and activation of lamivudine might be responsible for the suboptimal decline of HBV DNA in some patients. Therefore, we studied in chronic hepatitis B patients the availability of lamivudine in blood after a standard oral dose. With these data we have a tool to exclude a problem in the first step of the pharmacokinetic process.

Methods and Patients

Methods

Group A. Eight patients were evaluated for 24 hours after oral intake of a single dose of lamivudine 150 mg in order to obtain a lamivudine plasma reference curve. Patients fasted overnight and blood was withdrawn during 24 hours at t=0, 15, 30, 45, 60 and 90 minutes and 2, 3, 4, 6, 8, 12, 18 and 24 hours after intake of lamivudine.

Group B. In a group of nine patients in which the viral decline during lamivudine 150 mg therapy (6) was studied in detail, the lamivudine concentration in a serum sample taken 6 hours after start of lamivudine therapy was assessed. The pharmacokinetic reference curve was based on plasma samples. Therefore, the agreement between plasma and serum results was ascertained in 9 randomly selected patients on lamivudine who visited the outpatient clinic (group C).

High Pressure Liquid Chromatography (HPLC) of Lamivudine in Plasma and Serum

Lamivudine in plasma and serum was assayed with a HPLC-method slightly modified from Harker et al. (7). In short, the following procedure was used.

Sample extraction is performed using a solid phase extraction method (Bond Elute Verify LRC; 10 cc/130 mg, Varian Inc., Harbor City, CA, USA), after activation of the column with subsequently 2 ml of methanol and 2 ml of acetic acid 1%. Next, a mixture of 1 ml of plasma and 1 ml of acetic acid 1% is applied to the column with a pressure of 5 mm Hg for at least 2

min. The column is consecutively washed and dried with distilled water, methanol/acetic acid 10% (9:1) and distilled water again. Desorption is carried out four times with 0.5 ml of methanol / ammonia 25% (9:1) under a low vacuum. The four fractions are collected and evaporated to dryness with a gentle flow of nitrogen at 40 °C and subsequently suspended in 300 ul of the mobile phase by vortex-mixing. Separation of the mixture is performed by HLPC, equipped with a BDS Hypersil C18 column (250 x 4.6 mm ID; 5 um), using a mixture of methanol (40 ml), acetonitrile (5 ml), glacial acetic acid (0.5 ml) and 0.1 M ammonium acetate in water (455 ml) as the mobile phase at a flow of 1 ml/min and at a temperature of 40 °C. Quantification was based on UV-detection at 270 nm, calibrated with a range of external standards in plasma, that were processed the same way.

Intra- and inter-assay variability
Eight calibration standards of lamivudine with a concentration ranging from 0.1 mg/l to 7.5 mg/l were analysed simultaneously six times (intra-assay variability) expressed as the average accuracy with percent of the deviation from the nominal concentration; the procedure was repeated on three separate days (inter-assay variability) expressed as a coefficient of variation.

Correlation between lamivudine levels in plasma and serum
The concentrations of lamivudine in serum and plasma were compared by means of a linear plot as well as a Bland and Altman plot (8).

Modeling of pharmacokinetic data
From the 24 hours pharmacokinetic curves, the average area under the curve (AUC), the half-life of lamivudine ($t_{½}$), t_{max} and C_{max} were calculated. Lamivudine concentrations were fitted with the TOPFIT pharmacokinetic program (9) using a 1-, 2- and 3-compartment model using four weightings (1, $1/\sqrt{y}$, $1/y$ and $1/y^2$). The Akaike criterion (10) was used to establish the best fit of our data.

Results

Patient characteristics of group A, B and C are shown in table 1. In group A, a considerable part of patients were in an advanced stage of liver-disease as indicated by decompensated liver cirrhosis.

Table 1 Patient characteristics

	Group A (n=8)	Group B (n=9)	Group C (n=9)
Age (median;range)	37 (17-60)	28 (22-51)	29 (17-57)
Male/female	7/1	7/2	6/3
Cirrhosis	4	1	0 (n=8)
Additional medication	pt 3: ferrofumarate pt 5: furosemide, aldactone pt 7: methotrexate pt 8: data missing	pt 4: oral contraceptive	pt 1: pantozole pt 3: clinoril, cough-medicine, doxazosine, losartan, atorvastatine, insuline pt 4/9: paracetamol

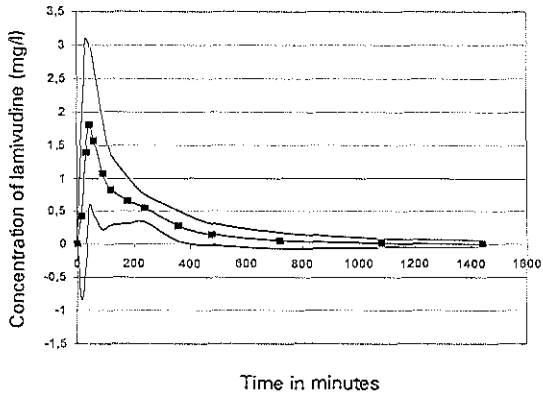
The lower limit of detection of the HPLC-assay was determined at 0.005 mg/l and the lower limit of quantification at 0.1 mg/l. The higher limit of detection was arbitrarily determined at 7.5 mg/l. All calibration curves were linear between 0.1-7.5 mg/l with a variance between –15% to +10% in this range. A variety of drugs, which were co-administered frequently to these patients, did not interfere with the extraction and detection procedure.
The intra-assay variability showed an accuracy of 80-95% which is comparable with data described in the literature (7). The inter-assay variability was concentration dependent, 3-16.6% (Table 2). Recovery of lamivudine in spiked plasma samples compared with non-processed standard solutions was approximately 80%.
The relation between the concentration of lamivudine in plasma and serum was linear as observed by a line with a slope of 0.997 and an intercept at (0,0). The Bland and Altman plot showed a mean of the difference between the serum- and plasma- level of 0.02 mg/l (s.d. ± 0.0411).

Table 2 Intra-assay and inter-assay variability.

	Intra-assay variability			Inter-assay variability		
Theoretical value (mg/l)	Mean (n=6)	Standard deviation	%Coefficient of variation	Mean (n=6)	Standard deviation	%Coefficient of variation
0.1	0.096	0.009	9.4	0.094	0.016	16.6
0.21	0.189	0.009	4.8	0.19	0.018	10
0.56	0.495	0.022	4.4	0.487	0.026	5.4
1.04	0.875	0.013	1.5	0.882	0.043	4.9
1.53	1.293	0.018	1.4	1.296	0.05	3.9
2.18	1.819	0.11	6	1.884	0.102	5.4
3.24	2.583	0.037	1.4	2.69	0.13	5
7.5	6.177	0.18	2.9	6.22	0.18	3

For group A, a mean AUC of 4994 mcg/l.h (s.d. 1524), a mean t_{max} of 42 minutes (s.d. 11) and a mean C_{max} of 1.9 mg/l (s.d. 0.70) were calculated (Fig 1).
If we compare the 6 hours serum concentration of lamivudine in group B (median 0.35 mg/l; range 0.28-0.52) with the same time point in group A (median 0.32 mg/l; range 0.15-0.48) these concentrations are within the same range.

Fig 1 Pharmacokinetic reference curve (± 2xs.d.) based on 8 chronic hepatitis B patients treated with lamivudine 150 mg. once daily (group A).



Discussion

If the inhibitory effect of lamivudine on HBV replication is studied with a sensitive assay with a dynamic range between 400-10^9 geq/ml, a wide variation in response between individual patients is observed. In a previous study, we showed that this could only in part be explained by the emergence of a mutation in the catalytic site of the polymerase gene of the hepatitis B virus (4). In this study we made a first step in further exploration of other mechanisms which might explain the variability of response to lamivudine.

The pharmacokinetic process of any drug, including lamivudine, is characterised by a sequence of events: absorption, distribution, metabolism and elimination. Lamivudine is highly soluble, dissolves rapidly once in the stomach and is being absorbed in the small intestine by passive diffusion. Food reduces the rate of absorption but not the extent: t_{max} is prolonged, c_{max} is reduced and the AUC is not altered (11). The absolute bio-availability is reported to be around 80% with a mean volume of distribution of 1.3 l/kg, indicating considerable distribution into deeper tissues (12). In chronic hepatitis B patients, lamivudine acts in the liver, the target organ for viral replication. Lamivudine probably enters hepatocytes through active uptake by pyrimidine nucleoside transporters (13,14). In the cytoplasm of the hepatocyte, lamivudine is phosphorylated to the mono-, di- and triphosphate by deoxycytidine kinase, cytidine monophosphate kinase and pyrimidine nucleoside diphosphate kinase respectively. The diphosphate is present in highest concentrations inside the hepatocyte and the conversion of the di-phosphate to the tri-phophate is the rate-limiting step (15). This extensive bio-activation makes the drug prone to individual differences between patients. Only 5-10% of lamivudine is metabolised to a trans-sulphoxide metabolite and excreted in urine, around 70% of the drug is excreted unchanged in urine (16).

In this study, we modified and validated the HPLC assay for detection of lamivudine in plasma. Only few data on pharmacokinetics of lamivudine in compensated chronic hepatitis B patients have been published (16). Our pharmacokinetic parameters are comparable to the published data. Measurement of levels of lamivudine in daily practice may be useful for two purposes. If the level is within the normal range, this ascertains that patients have been compliant with therapy on the one hand and that on the other hand absorption, the first pharmacokinetic step, is adequate.

As can be observed from our data, levels of lamivudine in plasma 6 hours after intake of lamivudine (group B) are in the same range of patients in group A at 6 hours. These data however, should be interpreted with caution, since group characteristics vary. Recent studies have stressed the potential influence of co-administered drugs on lamivudine kinetics. This is either caused by the increase of phosphorylation of lamivudine (e.g. hydroxyurea, methotrexate) (17) or because of reduction of the excretion ratio of lamivudine in urine (e.g. trimethoprim) (18).

Our kinetic data show that the lamivudine concentrations in group B are well above the *in vitro* IC_{50} (16) even 5 hours after the maximum concentration in plasma has been reached. These data also show that all patients have been compliant and that absorption was adequate. Plasma levels have been measured after intake of the first dose of lamivudine but these levels may change during long-term therapy. Previous data do not indicate that lamivudine accumulates during long-term application, but these data ascertain sufficient levels of lamivudine above the *in vitro* IC_{50} throughout the 24-hour period (16). In contrast, in 7 out of 8 patients of group A in our study levels of lamivudine 24 hours after intake are undetectable. This could necessitate re-opening of the discussion on twice daily dosing. However, this may not even be an issue, since we feel that more focus should be put on levels of lamivudine-phosphates inside hepatocytes. Half-life of lamivudine tri-phosphate in human lymphocytes infected with the Human Immunodeficiency Virus (HIV) have been calculated to be substantially longer (10.5-15.5 hours) than lamivudine itself in serum (19). Conflicting data on the half-life of lamivudine-triphosphate in hepatocytes have been published (3.6-8 hours in primary duck hepatocytes (20) versus 17-19 hours in HepG2 cell lines (16)). Therefore, research into human hepatocytes is needed both to address the dosing issue, as well as to better understand the differences between individual patients.

Absorption of lamivudine is a passive process and may therefore be the least important reason for variation in response to lamivudine between patients. In contrast, uptake of lamivudine in hepatocytes is most likely an active process, and lamivudine is phosphorylated inside hepatocytes. Phosphorylation is mediated by host enzymes and the efficacy of the process from parent drug to active tri-phosphate and persistence of the active tri-phosphate in the hepatocyte may vary between individual patients due to genetic polymorphism. Therefore, in order to be able to explain differences in viral decline between patients, further exploration into the patient to patient differences is needed.

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5 A

ANTIVIRAL TREATMENT FOR HUMAN IMMUNO-DEFICIENCY VIRUS PATIENTS CO-INFECTED WITH HEPATITIS B VIRUS: COMBINED EFFECT FOR BOTH INFECTIONS, AN OBTAINABLE GOAL?

L.M.M. Wolters[1], H.G.M. Niesters[2], R.A. de Man[1], S.W. Schalm[1]

Department of [1]Gastroenterology & Hepatology and [2]Virology,
University Hospital Rotterdam, The Netherlands

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Summary

A large percentage of HIV-1 infected patients have serological evidence of a past or present HBV infection,. Long-term survival is increasing for HIV-patients because of highly active antiretroviral therapy. Therefore, the chronic hepatitis B infection may become an important determinant of disease outcome in these co-infected patients.
We describe two HIV/HBV co-infected patients who were treated with extended antiviral therapy, initially indicated for the HIV infection. Lamivudine, a suppresser of viral replication in both infections, was one of these antiviral drugs. One patient showed a severe rebound of the HBV after withdrawal of lamivudine, the other patient developed a mutant hepatitis B virus after 18 months of treatment. This mutation was exclusively induced by lamivudine.
These patients show that, with improved HIV-related survival, the HBV infection should be monitored carefully, thereby enabling the physician to interfere with therapy when necessary.

Introduction

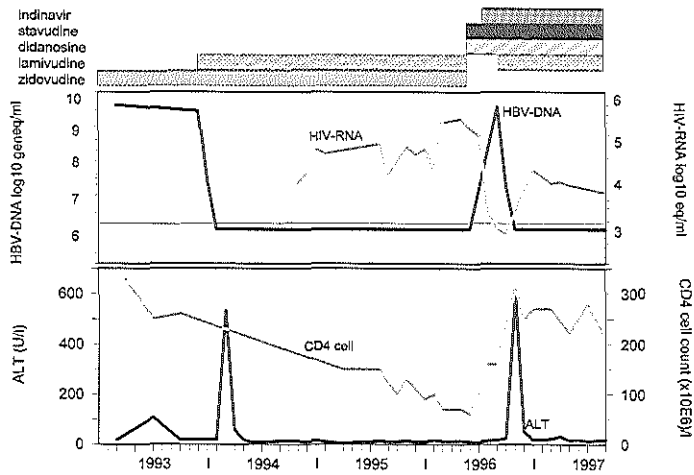
About 80% of Human Immunodeficiency Virus (HIV)-1 positive patients have serological evidence of past or present hepatitis B virus (HBV) infection (1). With the introduction of triple therapy for HIV-1 infected patients and the expectation of markedly prolonged survival, the consequences of chronic HBV infection in HIV-1 infected patients are becoming clinically relevant and a determinant of survival.
Lamivudine, the (-) enantiomer of 2'-deoxy-3'-thiacytidine, is virus suppressive in both HIV-1 infection and HBV infection (2,3). In addition, antiretroviral therapy may restore immunity through suppression of HIV-1 RNA and repopulation of the immune system. This adds to eradication of the HBV (4). Furthermore, nucleoside analogues used for HIV-1 infections, have been reported to cause lactic acidosis and liver steatosis (5). Special attention should be paid to these associated effects in patients with manifest liver disease, especially cirrhosis, as hepatic failure might be prevented.
Lamivudine can induce mutations in the YMDD motif of the HBV DNA polymerase gene in both HIV-1 and HBV (6-9). Withdrawal of lamivudine is usually followed by reactivation of HBV infection, detectable by rising HBV DNA levels. In some patients this surge of viral replication is followed by a hepatitis flare with elevated ALT levels (10).
Based on our experience in two patients co-infected with HIV-1 and HBV, we would like to focus attention on two syndromes associated with the use or withdrawal of lamivudine.

Case history

Patient A, a 49-year old man, initially visited our clinic in March 1990. Routine laboratory testing before surgery revealed liver function disorders. Subsequently he was found to be HBsAg positive and HBeAg positive. In addition to the HBV infection a HIV-1 infection was diagnosed. On physical examination no major abnormalities were detected, except a genital herpes-infection. Laboratory testing showed: no abnormalities other than AST 41 U/l (5-30 U/l); ALT 81 U/l (5-30 U/l) and CD4 cell count 0.46×10^9/l ($0.5\text{-}1.57 \times 10^9$/l). Serum markers of HBV infection: HBsAg positive; anti-HBc positive; HBeAg positive; anti-HBs negative; anti-HBe negative (IMx, Abbott, Chicago, USA) and HBV DNA 2.56×10^9 genome equivalents (geq)/ml (Eurohep standard (11), measured as 275 pg/ml in Genostics assay, Abbott, USA). Anti-hepatitis C virus (HCV) negative; anti-hepatitis D virus (HDV) negative; anti-cytomegalo virus (CMV) negative. A liver biopsy showed a chronic persistent hepatitis with mild periportal fibrosis.
Because of a decline of CD4 positive cells, AZT monotherapy was started in February 1992. Lamivudine was added in December 1993 (Fig. 1). During this treatment, the HBV infection became inactive according to non-detectable HBV DNA and HBeAg. The loss of HBeAg was associated with a hepatitis flare. HIV-1 RNA levels (Roche Monitor 1.0, Somerville, New Jersey, USA) however, remained high (> 1×10^4 geq/ml). In 1996, therapy was changed to stavudine, didanosine and indinavir triple therapy; lamivudine was withdrawn. HIV-1 RNA levels dropped markedly, even below detection levels, but stabilized around 10^3 geq/ml. CD4 cell counts increased from 0.16×10^9/l to above 0.2×10^9/l. Three months after discontinuation of lamivudine, HBV DNA rose to 3.7×10^9 geq/ml (measured as 12.010 pg/ml in Digene assay, Murex, UK), accompanied by a rise of HBeAg. Five months after cessation of lamivudine, ALT had risen to more than 600 U/l. At this moment HBV DNA levels had become undetectable by liquid hybridization again after the reintroduction of lamivudine. This was followed by a rapid decrease to normal ALT levels in December 1996. At March

1998 the HBV infection was still suppressed (HBV DNA negative by PCR, HBeAg negative), and serum ALT normal.

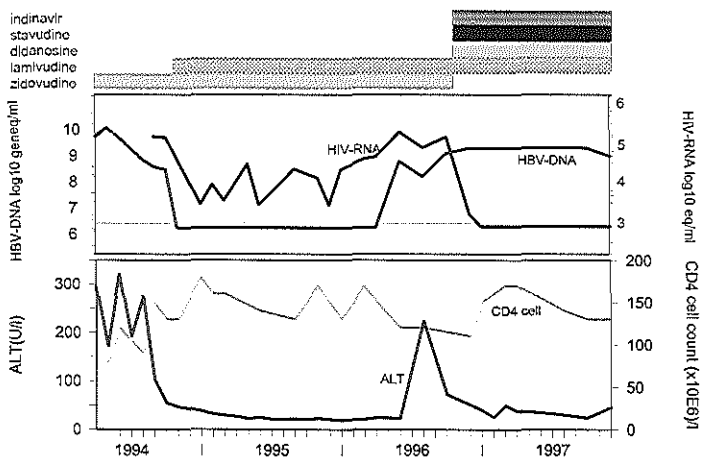
Fig 1 HIV-1 HBV co-infection. Patient A. The profound virus suppressive effect of lamivudine and the hepatitis episode associated with HBeAg loss after withdrawal of lamivudine is illustrated.



In March 1994, patient B, a 48-year old man was admitted to our hospital because of marked elevation of his liver enzymes. He was found to be HBV and HIV-1 positive.
On physical examination no significant abnormalities were detected. Laboratory results were: platelets $102x10^9$/l (140-360x10^9/l); CD4 cell count $0.08x10^9$/l (0.5-1.57x10^9/l); prothrombin time 13.0 seconds (9.3-12.3 seconds); bilirubin 20 µmol/l (4-14 µmol/l); alkaline phosphatase 84 U/l (25-75 U/l); γGT 71 U/l (5-35 U/l); AST 249 U/l (5-30 U/l); ALT 295 U/l (5-30 U/l); albumen 32 g/l (36-48 g/l); IgG 36.3 g/l (8-18 g/l); HBsAg positive; HBeAg positive; anti-HBc positive; anti-HBe negative (IMx, Abbott, Chicago, USA); HBV DNA $2.77x10^9$ geq/ml (measured as 277 pg/ml in Genostics assay, Abbott, USA); anti-hepatitis A virus (HAV) (IgM) positive; anti-HCV negative; anti-HDV negative; anti-HIV-1 positive.
The liver biopsy showed cirrhosis, chronic moderate active hepatitis with lobular involvement and collapse. The ultrasound of the upper abdomen showed hepatosplenomegaly and ascites.
Because of his low CD4 cell count, priority was given to HIV-1 therapy. Treatment was started with zidovudine 250 mg bd and co-trimaxol 480 mg od, half a year later lamivudine 300 mg bd was added. The medication was tolerated well.
During lamivudine therapy, HBV DNA fell below detection level in November 1994 (Fig. 2). HBeAg quantified with a Paul Ehrlich Standard, as reference and expressed in PEU (Units) declined from 487 PEU/ml to 0.9 PEU/ml in October 1995. HBe seroconversion did not occur. Concordant with the decline in virus levels, transaminase activity also declined to normal levels (below 30 U/l).
One and a half years after starting lamivudine (mid 1996), while the patient was still taking lamivudine and zidovudine, a sudden rise in HBV DNA and HBeAg levels, followed by a rise of ALT to levels above 200 U/l was documented. Sequencing of the YMDD motif of the C-domain of the DNA polymerase of HBV showed a mutation at position 552 at which

methionine was replaced by valine, indicating lamivudine resistance. Lamivudine was continued as ALT levels had fallen spontaneously and there was fear of lamivudine withdrawal hepatitis in a patient with cirrhosis. Because of continued elevated levels of HIV-1 RNA up to $9x10^4$ geq/ml, in October 1996 zidovudine was replaced by didanosine, stavudine, indinavir; the dose of lamivudine was reduced to 150 mg bd. Bilirubin rose markedly after the change in therapy which was attributed to interference of indinavir with the bilirubin metabolism; stable levels of prothrombin time, albumin and bile acids excluded progressive liver damage. ALT levels continued to fall and were back to normal at the beginning of 1997. The response of HIV-1 to the present medication is satisfactory with HIV-1 RNA below detection levels of quantitative PCR ($<5.0x10^2$ geq/ml), but only a marginal rise in CD4 cell count to $0.13x10^9$/l was observed. An active HBV infection with levels of HBV DNA up to $1.0x10^9$ geq/ml and HBeAg up to 4120 PEU/ml is still present, apparently without a detectable immune response.

Fig 2 HIV-1 HBV co-infection. Patient B. The profound hepatitis B virus suppressive effect of lamivudine and the reappearance of HBV DNA (lamivudine resistance), followed by a hepatitis flare is illustrated.



Discussion

Chronic HBV infection does not influence the progression of HIV-1 to AIDS (1,12). In contrast, HIV-1 does influence the course of a HBV infection. Acute hepatitis B progresses to chronic hepatitis B in 5-10% of HIV-1 negative adults; in those who are positive for HIV-1 the chronicity rate has been reported to be 40% (1). Chronic hepatitis B patients who are HIV-1 infected have a less pronounced tendency to show a spontaneous decrease in viral replication than those who are HIV-1 negative (12,13). In addition, antiretroviral therapy can restore immunity and induce recovery from HBV (4).
Our study shows that patients with HIV-1 HBV co-infection undergoing multiple treatments with nucleoside analogues can manifest hepatitis flares associated with drug induced resistance or with withdrawal.

A lamivudine withdrawal hepatitis flare, as seen in patient A, with ALT levels over ten times the upper limit of normal, occurs in 16% of immunocompetent patients. Usually these flares pass without clinically significant symptoms, in a minority of the patients jaundice is seen (14). Re-institution of lamivudine therapy reduces viral replication rapidly; simultaneously, the activated cytotoxic T cells can eliminate the remaining hepatocytes that express viral antigen. In our patient, suppressed viral replication with elevated immune response led to undetectable HBV DNA by PCR and loss of HBeAg.
Patient B became resistant to lamivudine reflected in a sudden rise of HBV DNA after 14 months of treatment. In immunocompetent patients, resistance to lamivudine develops in 39% (actuarial cumulative incidence) at one year (7). In HIV-1 HBV co-infected patients, lamivudine resistance to HBV has not been described in the cohort of 40 patients with a progressive HIV-1 infection, co-infected with HBV treated with lamivudine for 12 months (15). Insensitivity of the HBV for lamivudine has been described in liver transplant recipients who showed a recurrence of viral replication starting after six months of lamivudine treatment (16-19). A mutation in the highly conserved YMDD motif of the reverse transcriptase gene is described to be the cause of the decreased sensitivity to this drug. In patient B, substitution of valine for methionine at position 552 of the YMDD motif in the C-domain, which is explicitly linked to the leucine to methionine mutation at position 528 in the B-domain of the polymerase gene, was seen. After stopping lamivudine, the dominant virus population returned to the wild type, with accelerated viral replication and possible risk of hepatitis flare.

With the increased efficacy of triple therapy causing prolonged survival of HIV-1 infected patients, HBV infection may become an important determinant of disease outcome in these patients. Overlap in therapy for these two viral infections should be monitored carefully since change in therapy because of non-response to lamivudine of one disease (HIV infection) might cause reactivation of the other (hepatitis B).

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5 B

DEVELOPMENT OF HEPATITIS B VIRUS RESISTANCE FOR LAMIVUDINE IN CHRONIC HEPATITIS B PATIENTS CO-INFECTED WITH THE HUMAN IMMUNODEFICIENCY VIRUS IN A DUTCH COHORT

L.M.M. Wolters[1], H.G.M. Niesters[2], B.E. Hansen[3], M.E. van der Ende[4], F.P. Kroon[5],C. Richter[6], K. Brinkman[7], P.L. Meenhorst[8], R.A. de Man[1]

Departments of [1]Gastroenterology & Hepatology; [2]Virology; [3]Epidemiology & Biostatistics [4]Internal Medicine, University Hospital Rotterdam; [5]Leiden University Medical Center, Leiden; [6]Rijnstate Hospital, Arnhem; [7]Onze Lieve Vrouwe Gasthuis, Amsterdam; [8]Slotervaart Hospital, Amsterdam, The Netherlands

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Summary

Introduction With the introduction of HAART, the HIV-1 has turned from a lethal into a chronic infection in the majority of patients. In homosexual populations, twenty percent of HIV-1 infected patients suffer from a chronic HBV infection, which may eventually lead to complications of the liver disease because of prolonged survival. Lamivudine is effective in reducing both HIV-1 and HBV viral replication. However, resistance for lamivudine may complicate the course of the HBV disease in HIV-1-infected patients. We, therefore, conducted a retrospective study in HIV-1-HBV co-infected patients on lamivudine therapy.
Patients and methods All HIV-1-HBV co-infected patients who were treated with lamivudine for over 6 months in 5 major referral clinics in the Netherlands with HBV DNA above $2.0x10^5$ geq/ml at baseline, were evaluated. Retrospectively, the course of HBV DNA in available serum samples was established. If HBV DNA was detectable with the sensitive PCR-assay, YMDD-analyses of the polymerase gene of the hepatitis B virus was executed with the INNO-LiPA-DR-strip.
Results Forty-six patients were evaluated. The median level of HBV DNA at start of lamivudine therapy was $1.31x10^9$ geq/ml (range $3.5x10^5$-$2.0x10^{10}$, n=43). Of three patients no baseline sample was available, but since HBV DNA was still above $2.0x10^5$ geq/ml at week 3, 7 and 11, these patients were included. Median duration of lamivudine therapy was 97 weeks (range 27-263). The percentage of detected mutations was 25% and 52% at one and two years, respectively. Twenty-two patients ultimately developed a mutation. Both baseline Body Mass Index and the decrease in CD4 cell count as a time dependent factor were significantly related to the emergence of mutations. In 10 out of 12 evaluated patients, HBV DNA levels returned to baseline level or even above baseline level after the development of mutant virus. One patient (5%) developed a flare of serum transaminases (ALT>10xULN) 24 weeks after first detection of variant virus.
Conclusion There is a linear time-dependent appearance of HBV mutations for lamivudine in our population. In a minority of patients (5%), development of a mutation was followed by a significant elevation of serum transaminases. A decline in CD4 cell count, which may indicate less response to HAART, induces a faster emergence of mutations. Close surveillance of HBV co-infected patients on therapy may be indicated due to the prolonged survival of HIV-1 patients.

Introduction

Both the Hepatitis B Virus (HBV) and the Human Immunodeficiency Virus-1 (HIV-1) are transmitted similarly through sexual contact and blood-blood contact. Therefore, the majority of HIV-1 infected patients show serological evidence of a resolved or still present hepatitis B infection (1, 2).
During the early years of the HIV-1 epidemic, antiretroviral therapy for the HIV-1 infection in HIV-1-HBV co-infected patients was given priority since this disease was the most important factor for survival. However, since 1997 combination therapy of nucleoside analogues (most often including lamivudine) with protease inhibitors or non-nucleoside reverse transcriptase inhibitors (3, 4), Highly Active Anti-Retroviral Therapy (HAART) is standard of care, resulting in sustained decreases of plasma HIV-1 RNA levels and a decrease in mortality in a large proportion of patients. As a result, the chronic hepatitis B infection in these patients and its potential complications might become more prominent.
Both HIV-1 and HBV use a virus specific polymerase which mediates reverse transcription. This polymerase incorporates lamivudine into the proviral chain and thereby induces chain termination. As a result, lamivudine is capable of inhibiting viral replication of both HIV-1 and HBV (5-9). However, lamivudine monotherapy can induce the emergence of a mutation in the catalytic site of the polymerase gene (YMDD), resulting in reduced viral suppression (10-15). The pattern of development of resistance for lamivudine in HBV is more gradual than in HIV-1. Nevertheless, previous studies in immunocompetent chronic hepatitis B patients show a cumulative incidence of 14-39% at one year (8, 16) increasing to 38-60% at two years of therapy (17, 18). In HIV-1-HBV co-infected patients a different pattern might be observed because of a suppressed immune status as reflected by decreased levels of CD4 positive lymphocytes.
We conducted a retrospective study in 5 major referral clinics in the Netherlands to investigate the emergence of resistance for lamivudine in HIV-1-HBV co-infected patients who were treated with HAART including lamivudine.

Patients and methods

Five major referral hospitals in the Netherlands participated in this study. Eligible patients were HIV-1-HBV co-infected patients with a proven chronic hepatitis B infection, as observed by HBsAg positivity in serum for more than six months or by HBsAg positivity, negativity for anti-HBc IgM antibodies combined with active viral replication. All patients had detectable HBV DNA with the Digene II hybrid capture plate assay and were treated with lamivudine 300 mg od for at least six months.
All patients visiting the outpatient clinic and who were treated with lamivudine for at least 6 months were evaluated from the first day of lamivudine therapy until the last available serum- or plasma-sample while on treatment, or the last visit in 1999. HBV-DNA levels were assessed on a frequent basis.
In this retrospective study, frozen samples were evaluated for a mutation in the polymerase gene of the HBV at one year if they expressed active viral replication of the HBV as measured by the quantitative PCR-assay. Patients in which HBV DNA levels were below 1000 geq/ml at yearly intervals were considered to have not developed a variant HBV strain. Of these patients no mutation analysis was performed because of the limited amount of virus present. If a mutation in the HBV polymerase was observed at 1 year or 2 years, additional analyses were performed retrogradually.

CD4 cell count and CD8 cell count which were assessed within 6 months before start of lamivudine therapy were regarded as baseline values. Values of serum transaminases and bilirubin were considered baseline levels if obtained within three months before start of lamivudine therapy. Changes in CD4 cell count during therapy were documented.

Virology
HBV DNA was quantified with a Digene II Hybrid Capture plate (HCS) assay (with a limit of detection of $2.0x10^5$ geq/ml). If HBV DNA was below the limit of detection of the HCS, the viral load was assessed using the HBV Monitor assay (Roche Diagnostics, Almere, The Netherlands; lower limit of detection of 1000 geq/ml). Both assays were calibrated using the EUROHEP standard (19). IgM anti-HBc was performed with a Micro-particle Enzyme Immuno Assay (AxSYM, Abbott, Chicago, IL).
HIV-1 RNA levels were determined at the different sites with a commercial assay with a lower limit of detection of 500 geq/ml or less. Antibodies to the hepatitis C virus (aHCV), antibodies to the hepatitis D virus (aHDV) and HDV RNA were determined as previously described (20). HCV RNA was detected with the Cobas Amplicor HCV RNA 2.0 assay (Roche Diagnostics, Almere, The Netherlands).
A Line Probe Assay (INNO LiPA Drug Resistant (DR) HBV, Innogenetics N.V., Ghent, Belgium) was used to determine the sequential occurrence of HBV mutations for lamivudine. The procedure was performed essentially as described by Stuyver et al (21). HBV DNA was isolated from serum using the High Pure Viral Nucleic Acid kit (Roche Diagnostics).

Statistics
The Kaplan-Meier method was applied to calculate the observed percentage of occurrence of mutations. The Cox-regression analysis was used to investigate the independent effect of the baseline factors sex, route of transmission of both viral infections, country of birth, centre, age, HBV DNA viral load, HIV-1 RNA viral load, CD4 cell count, CD8 cell count, CD4/CD8 ratio, ALT in relation to the Upper Limit of Normal (ULN), AST in relation to the Upper Limit of Normal (ULN), bilirubin and Body Mass Index (BMI) on the moment of occurrence of mutations. To study the effect of the changes in CD4 cell count during treatment on the occurrence of mutations, a Cox model with baseline CD4 cell count at baseline and changes of CD4 cell count per week as time dependent factor was used. Significance was defined as $p<0.05$.

Results

Patients
Forty-six patients were included in this study (Table 1). Twenty-nine patients had proven HBsAg positivity in serum for more than 6 months. Fifteen patients, of whom no previous blood samples were available before the first detection of HBsAg positivity, were negative for anti-HBc IgM while showing active viral replication. In one patient anti-HBc IgM status before start of therapy was not known, however, the HBV was still actively replicating more than six months later. Another patient was only weakly positive for anti-HBc IgM which is compatible with a chronic infection. Both patients were considered to be chronic hepatitis B patients. Patient characteristics are shown in table 1. The median level of HBV DNA in 43 patients before start of lamivudine therapy (at a median of 3 weeks (range 0-38 weeks) before start of therapy) was $1.31x10^9$ geq/ml (range $3.5x10^5$- $2.0x10^{10}$ geq/ml). Of three patients, no serum samples were available before start of lamivudine therapy; since HBV DNA was still

over $2.0x10^5$ geq/ml at 3, 7 and 11 weeks of lamivudine therapy, these patients were also included in the analysis.

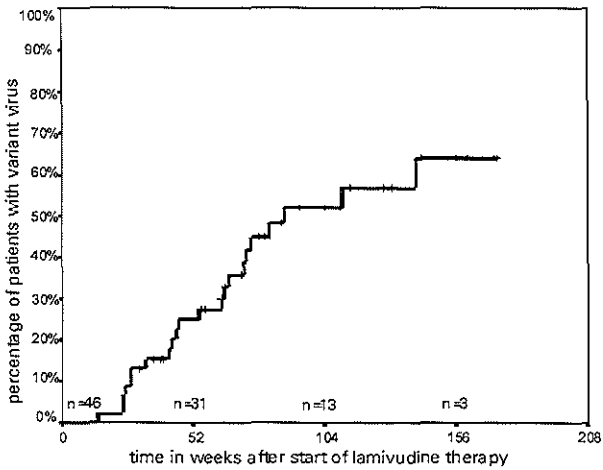
Table 1 Baseline characteristics (n=46)

Age median (range)		38 years (24-62)
Sex (M:F)		44:2
Race	Caucasian	33 (72%)
	African origin	6 (13%)
	South-American origin	7 (15%)
Route of transmission	Homosexual	33 (72%)
	Heterosexual	11 (24%)
	Intravenous drugs	2 (4%)
BMI (kg/m^2) n=42		21.8 (15.8-27.7)
HBV DNA (geq/ml, median; range) n=43		$1.31x\ 10^9$ ($3.5x10^5$-$2.0x10^{10}$)
HIV-1 RNA (geq/ml, median; range) n=35		$7.76x10^4$ (80-$2.58x10^6$ geq/ml)
ALT (xULN, median; range) n=36		1.8 (1-10.6)
ALT<ULN		n=8 (22%)
AST (xULN, median; range) n=41		2.1 (1-9.6)
AST<ULN		n=10 (24%)
Bilirubin (>ULN:<ULN) n=30		5:25
CD4 cells (>$200x10^6$:<$200x10^6$) n=41		17:24

At start of lamivudine therapy (150 mg of lamivudine twice daily), 23 patients (50%) started with a combination of up to four nucleoside analogues (zidovudine, didanosine, stavudine or zalcitabine) and one or two protease inhibitors (ritonavir, indinavir, nelfinavir or saquinavir); 18 patients (39%) received a combination of two or three nucleoside analogues and a minority of patients (5; 11%) received a combination of two or three nucleoside analogues and a non-nucleoside reverse transcriptase inhibitor (NNRTI, nevirapine). Fifteen patients were pre-treated with antiretroviral therapy at the moment of start of lamivudine therapy. Forty-three patients were negative for antibodies to the hepatitis C virus (aHCV). Two patients had detectable antibodies for HCV, however, these patients did not show active HCV replication as shown by HCV RNA PCR negativity. In one patient, HCV status was not known. Thirty-nine patients had not been in contact with the hepatitis D virus (HDV) as shown by aHDV negativity. Five patients were positive for aHDV; in 3 of these patients HDV RNA was also positive. These patients were included in the analysis because they all exhibited active viral replication. The anti-HDV status was not known in 2 patients.

The median duration of lamivudine therapy was 97 weeks (range 27-263). However, the median duration until the last observation of HBV DNA during lamivudine therapy was 91 weeks (range 14-263). The median CD4 cell count at baseline was $120x10^6$ cells/µl (n=41; range $1x10^6$-$720x10^6$ cells/µl). During therapy, 14 out of 24 patients (58%) with a CD4 cell count below 200 cells/µl showed an increase of CD4 cells to above 200 cells/µl, indicating that 10/40 (25%) patients had a CD4-cell count below 200 cells/µl throughout the study period.

Fig 1 Kaplan-Meier curve representing the observed occurrence of mutations in the polymerase gene of HBV during lamivudine therapy.



During therapy, 13 patients became PCR negative; 18 patients still had actively replicating virus (HBV DNA detectable by PCR) but HBV DNA below baseline levels and 15 patients showed a rebound in viral replication after an initial decline, 2 of whom initially became PCR negative. In our population of 46 patients, the percentage of observed YIDD or YVDD mutations (a substitution of methionine for isoleucine or valine in the C domain of the HBV polymerase) against lamivudine was 25% (95% CI 12-38%) at one year and 52% (95% CI 35-69%) at two years (Fig 1). These percentages are based on the duration of lamivudine therapy. Twenty-two patients (48%) developed a mutation. The first observation of a mutation was at 14 weeks (median 88 weeks (95% CI 38-138 weeks)). In 10 out of 22 patients, the first observation of a mutation was a combination of either two mutants (YVDD and YIDD, n=2) or a combination of one or two mutants and wildtype virus (YVDD, YIDD, YMDD, n=8). In five patients who developed a mutation, we were capable of determining a pattern of consecutive mutations (Table 2). As can be observed from these data, all patients eventually developed a YVDD mutation, even though in patient 29, the YIDD mutation was still present aside the YVDD mutation at week 141. In 18/22 patients (82%), HBV DNA level was below or equal to baseline level at the moment of detection of a mutation. In 10/18 patients of whom we had follow-up samples after the development of variant virus –but still on therapy-, hepatitis B viral load returned to baseline level (n=2) or higher than baseline level (n=8). Only 1/22 (5%) patients developing a variant virus showed an increase of serum transaminases to levels over 10xULN detected 24 weeks after the first observation of the presence of mutant virus. This flare of serum transminases did not coincide with a distortion of liver functions as indicated by a normal prothrombin time (PT) and albumin. CD4 positive cell count at the time of the hepatitis flare was 440 cells/µl (normal range: 500-1700 cells/µl).

Table 2 Time dependent sequence of mutations in five patients

Patient	Initial mutation	week	Consecutive mutation	week
4	YIDD/YVDD	25	YVDD	42
19	YMDD/YIDD	24	YMDD/YIDD/YVDD→ YVDD	58
23	YMDD/YIDD/YV DD	45	YVDD	55
29	YMDD/YIDD/YV DD	33	YIDD/YVDD	50
30	YMDD/YVDD	43	YVDD	64

Effect of baseline factors on the time of emergence of a mutation

Sex, country of birth, route of transmission of the HBV and HIV-1, centre, age at baseline, baseline HBV DNA, HIV-1 RNA, ALT in relation to the ULN, AST in relation to the ULN, CD4-cell count, CD8-cell count, ratio of CD4- to CD8- cells, bilirubin and BMI were tested to investigate a possible relation to the time of emergence of a mutation. The BMI was the only significant baseline factor related to the time of emergence of a mutation (p=0.02) with a relative risk of 1.23 (Table 3). Extending the model with other covariates next to the BMI did not result in any other significantly related factors. The change in CD4 cell count per week during therapy included as a time-dependent factor was significantly related to the time of emergence of mutations: a decrease of $10x10^6$ cells/µl per week results in a relative risk of 1.29 (p=0.005) to develop a mutation (Table 3). In the multivariate analysis, including both baseline BMI and the time dependent decrease in CD4 cell count, these two covariates were independently related to the time of emergence of mutations.

Table 3 Relation of baseline factors to the time of emergence of a lamivudine induced mutation by Cox univariate regression analysis

Baseline factor	Relative Risk	95% Confidence Interval	p-value
Age	1.02	0.96-1.08	0.57
HBV DNA	1.3	0.86-1.96	0.22
HIV-1 RNA	0.86	0.49-1.52	0.61
CD4 cell count	0.75	0.30-1.87	0.19
CD4 cell count (>$200x10^6$:<$200x10^6$)	1.63	0.60-4.43	0.34
CD8 cell count	2.46	0.20-30.46	0.48
CD4/CD8 ratio	0.42	0.49-3.64	0.43
ALTxULN†	1.03	0.90-1.19	0.66
ASTxULN†	1.10	0.90-1.34	0.35
Bilirubin (<ULN† versus >ULN†)	1.70	0.59-4.94	0.33
Body Mass Index	1.23	1.03-1.46	0.02
Time dependent analysis			
Decrease of CD4 cell count of $10x10^6$ cells/week	1.29	1.08-1.53	0.005

† Upper Limit of Normal

Discussion

Mutations in the YMDD in the region of the C domain of the polymerase gene of the hepatitis B virus induce a less efficient incorporation of lamivudine in the proviral hepatitis B strand (22). In this study on HBV-HIV-1 co-infected patients, we observed a 25 and 52 percent occurrence of mutations at 1 and 2 years of lamivudine therapy, respectively. Since our study is a retrospective study and data were obtained from the available stored serum and plasma samples only, there might have been an underestimation of the actual occurrence of mutations in this patient group. Our data however, are in agreement with reports on the emergence of mutations during lamivudine therapy in immunocompetent Caucasian chronic hepatitis B patients (16) and liver transplant recipients on immunosuppressive therapy (23-26). Also, a cohort of French HIV-1-HBV co-infected patients (27) and a subpopulation of patients participating in the CAESAR study (28) showed similar results (50% of mutations at two year). The former study has a smaller sample size than our study population and in the latter study, only those patients with an initial response to lamivudine were evaluated for the emergence of mutations. However, also those patients with less favourable response to lamivudine may develop variant virus, therefore we decided to include all patients who were started on lamivudine therapy. Moreover, both studies apply direct sequencing techniques for detection of lamivudine resistance which is a less sensitive method than the InnoLiPA-DR-assay. With the sequencing technique, mutant virus can be detected if it accounts for more than 50% of the complete viral population whereas the InnoLiPA-DR-assay is capable of detecting only 5% of mutant virus within the mixed viral population.

The prevalence of mutations in any viral genome, possibly leading to resistance to antiviral drugs, is dependent on many factors. The level of viral replication, the error rate of the polymerase, the antiviral activity of the drug and the turnover of infected cells seem to be the most important factors that influence the mutation rate. In untreated immunosuppressed HIV-1-HBV co-infected patients, the response of the immune system on viral replication may be weak, reflected by high levels of hepatitis B replication in relation to less elevated serum transaminases. A small study in liver transplant patients with post-transplant recurrence of HBV infection, showed that baseline levels of HBV DNA and ALT were two independent predictors for development of lamivudine resistance (29). Our data do not support this finding: baseline HBV DNA and ALT were not related to the time of emergence of mutations. In contrast, a decrease in CD4 cell count as a time dependent factor results in a faster development of a HBV mutation. A decrease in the CD4 cell count could be related to less response to HAART which in turn could be caused by less response of the HIV-1 to the applied combination of antiviral drugs or a failing compliance. Both causes could result in a higher hepatitis B viral replication and faster development of HBV mutations due to a decreased immune system. Our finding could therefore be in agreement with the study by Borroughs et al. who describe a higher mutant growth rate in immunosuppressed liver transplant patients as compared to lamivudine treated immunocompetent patients (30). However, the mechanism on which faster viral replication in transplant patients may be based, is partly different from immunodeficient HIV-1-HBV co-infected patients due to the direct stimulation of viral replication by immunosuppressive agents (31). In case of a failing compliance, the alternate application and withdrawal of lamivudine may also add to the faster occurrence of mutations. Whatever the cause of the decrease in CD4 positive lymphocytes, patients with an unfavourable response to HAART should be monitored carefully.
In addition to the time dependent influence of the decrease of CD4 cells to the faster emergence of mutations, the BMI was found to be the only baseline factor predictive of the emergence of

mutations. Lamivudine exhibits a large volume of distribution (1.3 l/kg) which implies that lamivudine is transported to deeper tissues after absorption but it is not stored in fat (32). In a previous study in immunocompetent chronic hepatitis B patients, we observed that the level of lamivudine in plasma at day 1 of therapy was not related to viral decline during 4 months of lamivudine therapy (33). Since all patients in the present study received 300 mg of lamivudine, it should guarantee sufficient lamivudine plasma concentrations to obtain adequate intracellular levels to suppress the HBV replication. The relation of the higher BMI to the faster occurrence of mutations does not seem to be easily explained. However, from our data, we hypothesise that patients with a higher BMI might benefit from higher doses of lamivudine, but this needs to be studied more extensively.

Mutation analysis with the InnoLiPA-DR-assay is an elegant method for detection of YMDD variants in the polymerase genome. Since already 5% of the variant in the total viral population can be detected, the method is suitable for early detection of the most common mutations in the polymerase genome of HBV. In five patients who developed a mutation, we analysed several sequential serum samples. All patients showed a shift from an initial YIDD to a YVDD mutation. This sequence of mutations has been observed previously (13, 34) and it indicates that the valine mutation, which is explicitly linked to a mutation in the B domain at position 528, is probably the more stable mutation of the two.

In conclusion, in this HIV-1-HBV population in which patients have been treated with long-term lamivudine therapy there seems to be an almost linear occurrence of mutations. New antiviral drugs like entecavir and adefovir dipivoxil may be an option for this resistant patient population. HIV-HBV patients with less response to HAART therapy should be monitored more closely for the occurrence of mutations than patients who do respond well to therapy.

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5 C

EFFICACY OF FAMCICLOVIR TREATMENT IN CHRONIC HEPATITIS B PATIENTS WITH DIFFERENT MUTATIONS AT POSITION 552 OF THE DNA POLYMERASE GENE

L.M.M. Wolters[1], P. Honkoop[1], H.G.M. Niesters[2], R.A. de Man[1]

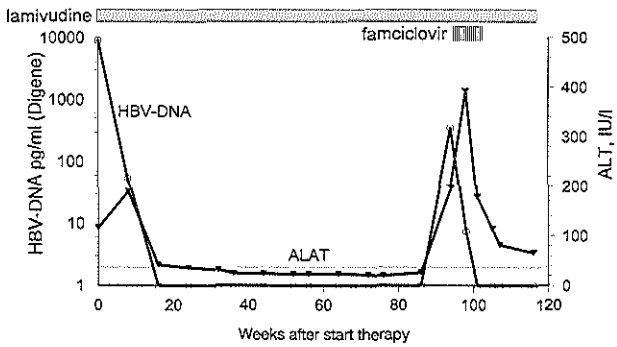
Department of [1]Hepatogastroenterology and [2]Virology, University Hospital Rotterdam, The Netherlands.

J Hepatol 1998;28:909-10

Standard Interferon alpha treatment is only efficacious in 33% of selected chronic hepatitis B patients (1). Therefore, there is a need for additional non-toxic therapies. Lamivudine is a potent antiviral drug which is able to suppress hepatitis B virus (HBV)-DNA in serum to undetectable levels (by liquid hybridization assay) in more than 80% of patients after 6 months of treatment (2). If, however, HBeAg does not become negative, treatment with lamivudine has to be continued. After prolonged treatment, resistance will evolve in up to 14% of the patients after 1 year (3). This is reflected in a rise in HBV-DNA and alanine aminotransferase (ALT) activity. In patients treated with lamivudine after liver transplantation, a point mutation in the YMDD-locus of the polymerase gene has been shown (4).
Four chronic HBV patients were treated with lamivudine for a median time of 60 weeks (range 30-86). They responded well and became HBV-DNA negative by liquid hybridization assay (Digene) within 4-18 weeks. After a median period of 56 weeks (range 26-72) they relapsed, as shown by a sudden increase in HBV-DNA, followed by a rise in ALT levels. Sequencing of the YMDD-motif of the C-domain of the polymerase gene in two patients, revealed a mutation: methionine at position 552 was replaced by isoleucine (M552I). In two other patients, methionine was replaced by valine (M552V). Because of the increase in ALT and HBV-DNA, we decided to evaluate famciclovir 500 mg three times a day for 4 weeks, in addition to continued lamivudine therapy. Famciclovir is, like lamivudine, a nucleoside analogue which inhibits DNA-polymerase. One patient became HBV-DNA negative concomitant with famciclovir (Fig 1). Three other patients showed a minimal decrease in HBV-DNA (0.1-0.4log). Two patients with M552V and one patient with M552I did not respond to famciclovir. Patients with the valine mutation at position 552 also have a second mutation at position 528 in the B-region of the reverse transcriptase part of the polymerase gene in which leucine is replaced by methionine. A virus with this mutation has previously been described to be resistant to famciclovir (5). The coexistence of these two mutations might stabilize the DNA-polymerase configuration (6). Resequencing of the polymerase gene of the patient who was initially found to have an isoleucine mutation and who did not respond to famciclovir, again showed M552I. The hypothesis in re-evaluating this patient, is that the isoleucine mutation represents a transitional state in which the isoleucine mutation coexists next to the valine mutation in serum. This series of events has been described in HIV patients who developed resistance to lamivudine (7). The transitional state has also been reported in immunocompetent lamivudine-resistant HBV patients, in whom YIDD is thought to be the temporal intermediate (6).
However, the HBV-DNA peak seen in patients might also be the expression of a breakthrough caused by the M552I. The rise in HBV-DNA is followed by inflammation, reflected by a sudden rise in ALT, which resolves this viremic state. In this scenario, famciclovir is not responsible for the rapid decline in HBV-DNA.

In conclusion, in one of two patients with a lamivudine-induced M552I mutation, a loss of HBV-DNA coincided with famciclovir therapy. No significant response to famciclovir was observed in two patients with a valine mutation at position 552.
If addition of famciclovir therapy is considered in lamivudine-treated patients, we think this should only be done after analysis of the underlying mutation in the polymerase region of HBV.

Fig 1 Response of HBV-DNA and ALT to lamivudine therapy. In this patient, at week 90, a mutant M552I was detected; the effect of famciclovir administration is shown in the graph.



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6

VIRAL DYNAMICS IN CHRONIC HEPATITIS B PATIENTS TREATED WITH LAMIVUDINE, LAMIVUDINE-FAMCICLOVIR OR LAMIVUDINE-GANCICLOVIR

L.M.M. Wolters[1], B.E. Hansen[2], H.G.M. Niesters[3], R.A. de Man[1]

Departments of [1]Gastroenterology & Hepatology, [2]Epidemiology & Biostatistics and [3]Virology,
University Hospital Rotterdam, The Netherlands

Submitted

Summary

Prolonged nucleoside analogue therapy has proved to induce reduction of viral replication and normalization of serum transaminases in the majority of chronic hepatitis B patients. However, from a theoretical point of view, monotherapy with lamivudine (a cytosine nucleoside analogue) will probably not result in eradication of hepatitis B virus (HBV). Extended prolongation of lamivudine therapy would be needed to clear the virus from the liver. The occurrence of mutations in combination with continuing low-grade viral replication in a number of patients will prevent elimination of the virus from the liver. However, combination therapy with more than one nucleoside analogue could possibly overcome the disadvantages of monotherapy.

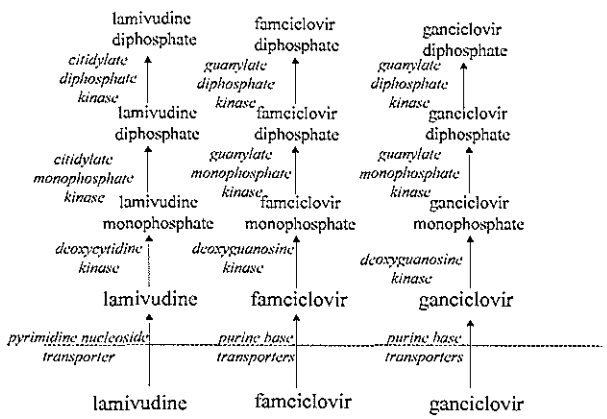
In this study, we report on 12 patients who were evaluated by means of a mathematical model during lamivudine monotherapy, lamivudine-famciclovir and lamivudine-ganciclovir therapy. The parameters representing blocking viral production, turn-over of free virus and turn-over of infected hepatocytes were not different between the treatment groups.

Although our study group is small, these combinations probably do not offer a major advantage over lamivudine monotherapy. Different combinations of nucleoside analogues need to be studied in order to obtain a major breakthrough for this treatment strategy.

Introduction

Lamivudine monotherapy has proven to be of benefit for chronic hepatitis B patients. Its main action is reduction of viral replication followed by normalization of serum transaminases and improvement of liver histology in the majority of patients (1-3). Especially for patients with an activated immune system, as reflected by elevated serum transaminases above 2x the Upper Limit of Normal (ULN), the chance of HBeAg seroconversion is enhanced (2, 4, 5). However, a considerable proportion of the patients continue to exhibit active viral replication after several months of lamivudine monotherapy (6, 7). Less than optimum viral suppression results in a continuing relatively high turnover of virus with an enhanced chance of induction of genetic diversity of the virus. Studies on therapies for Human Immunodeficiency Virus (HIV) patients have indicated that combining several nucleoside analogues may be a good way to postpone or maybe even prevent the emergence of mutations (8, 9). Since HBV and HIV both replicate by means of a viral polymerase and nucleoside analogue monotherapy results in resistance sooner or later, eventual treatment strategies will probably resemble HIV strategies.
In particular HBV patients who did not respond to previous lamivudine therapy may benefit from combination therapy with a compound with slightly different characteristics than lamivudine. Phosphorylation of different nucleoside analogues to the active nucleoside analogue tri-phosphate inside the hepatocyte is mediated by different enzymes (Fig 1). Moreover, tri-phosphates of different nucleoside analogues exhibit different mechanisms of action. Therefore, a combination regime may result in more pronounced suppression of viral replication, thus rapidly reducing the generation time of the virus and postponing or maybe even preventing the emergence of mutations.

Fig 1 Lamivudine, famciclovir, and ganciclovir: uptake into hepatocytes and phosphorylation into thetri-phosphate. Famciclovir is de-acytylated and oxidized to penciclovir before uptake into the hepatocyte.



In this randomized study, we evaluated the potential of a combination of a cytosine nucleoside analogue with a guanine nucleoside analogue versus standard lamivudine therapy. For one month, patients received either lamivudine monotherapy or lamivudine-famciclovir or lamivudine-ganciclovir combination therapy. The effect of these combination therapies was evaluated by means of dynamic description of viral decay, as previously used for lamivudine monotherapy (10).

Patients and methods

Eligible patients included men and women over 18 years of age who had a chronic hepatitis B virus infection defined by a biopsy-proven chronic hepatitis B or HBsAg positivity for more than 6 months. HBV DNA had to be $>1.5x10^6$ genome equivalents/ml (geq/ml). HBeAg status had to be stable as documented by HBeAg positivity or negativity for at least three months before the start of therapy. Inflammation of the liver had to be limited as expressed by serum transaminases below ten times the Upper Limit of Normal (ULN). Patients had to be negative for antibodies to the hepatitis C (HCV) or D virus (HDV) or the Human Immunodeficiency Virus (HIV). Patients with decompensated liver disease, liver diseases with other etiologies or other serious concomitant illnesses were excluded. The study was approved by the Medical Ethics Committee of the University Hospital Rotterdam. All patients had to give written informed consent.

Twelve patients were stratified according to previous lamivudine therapy and then randomly assigned to receive either lamivudine monotherapy (150 mg od), lamivudine (150 mg od) combined with famciclovir (500 mg three times a day) or lamivudine (150 mg od) combined with ganciclovir (1000 mg three times a day) for 4 weeks. Randomization was computer-generated and randomization labels were kept in sealed non-opaque randomization envelopes. All patients were screened for eligibility within four weeks before the start of treatment. Patients were admitted to the hospital for the first two days (day 0-2). Blood samples for detection of HBV DNA were drawn at screening, baseline and at t=6, 12, 18, 24, 30, 36, 42 and 48 hours. Subsequently, blood samples were obtained daily during the first week and on days 7, 10, 14, 21 and 28. Safety was assessed on a weekly basis.

HBV DNA measurement
HBV DNA was quantified with a Digene Hybrid Capture II tube liquid hybridization assay (calibrated on the EUROHEP standard (11)). If HBV DNA became undetectable with the Digene Hybrid Capture II tube liquid hybridization assay (limit of detection $2.0x10^5$ geq/ml) during lamivudine treatment, it was reassessed with the quantitative PCR (Roche, Amplicor Diagnostics, Almere, The Netherlands calibrated on the EUROHEP standard). This assay has a lower limit of detection of 1000 geq/ml.

Modeling
Viral decay was modeled with a bi-phasic model previously used to evaluate HCV patients on alpha interferon (12) and viral decay in HBV patients during nucleoside analogue therapy (10, 13, 14). Patients were fitted individually, and then group medians were calculated. A significant difference in parameters between the three treatment arms was obtained if $p<0.05$.

Results

Twelve patients were randomly assigned to one of the three treatment arms. Four patients received lamivudine monotherapy (group 1), two of these patients had previously been treated with lamivudine. Four patients were treated with lamivudine-ganciclovir combination therapy (group 2), three of whom had previously received lamivudine. In the third group, four patients were treated with lamivudine-famciclovir combination therapy (group 3), three of whom had previously been treated with lamivudine. Age at baseline was comparable between the three groups. The majority of patients were of Caucasian origin (Table 1). The median level of HBV DNA was $1.13x10^9$ geq/ml (range $8.6x10^7$-$4.01x10^9$), $2.47x10^9$ geq/ml (range $9.98x10^6$-$1.1x10^{10}$) and $2.54x10^9$ geq/ml (range $9.49x10^6$-$4.6x10^9$) for groups 1, 2 and 3, respectively. There was no significant difference in HBV DNA level between the three groups. All patients were aHCV and aHDV-negative. Eleven patients were negative for HIV. One patient in group 1 was not tested for the presence of antibodies to HIV. Median level of ALT in relation to the Upper Limit of Normal (ULN) was 1.6 (range 1.1-1.9), 1.5 (range 1-3) and 1.1 (range 1-1.2) for groups 1, 2 and 3, respectively). No significant differences between groups existed.

Table 1 Baseline characteristics

	Lamivudine monotherapy	Lamivudine-ganciclovir combination therapy	Lamivudine-famciclovir combination therapy
Age (years; median;range)	29 (21-56)	32 (25-41)	37 (22-47)
Race Caucasian	3	3	3
Asian	1	1	1
Sex (M:F)	2:2	4:0	2:2
Previous therapy with lamivudine	2	3	3
Median HBV DNA (geq/ml); range	$1.13x10^9$ ($8.6x10^7$-$4.01x10^9$)	$2.47x10^9$ ($9.98x10^6$-$1.1x10^{10}$)	$2.54x10^9$ ($9.49x10^6$-$4.6x10^9$)
Median ALT (xULN); range	1.6 (1.1-1.9)	1.5 (1-3)	1.1 (1-1.2)

Absolute viral decay during 1 month of therapy was not significantly different between the three groups (2.34 log (range 1.45-3.69), 2.39 log (range 1.21-3.30) and 2.07 log (range 1.78-2.28) for groups 1, 2 and 3 respectively. In two patients (1 in group 2 and 1 in group 3) normalization of serum transaminases occurred. Viral dynamic parameters did not reveal a difference in behavior between the three groups (Table 2). In the first phase of viral decay, which represents the turnover of free virus, there was a non-significant difference between the three groups; however, a somewhat faster turnover was found for the combination therapies (16 hours for the lamivudine monotherapy versus 12 hours and 10 hours for lamivudine-ganciclovir and lamivudine-famciclovir, respectively). The turnover of infected hepatocytes was most pronounced in the lamivudine-ganciclovir-treated patients. Effectiveness in blocking viral production was lowest in the lamivudine-ganciclovir group and highest and most consistent in the lamivudine-famciclovir group with all patients exhibiting more than 94% blocking of viral production.

Table 2 Different parameters calculated with the bi-phasic model

	Lamivudine monotherapy (median; range)	Lamivudine-ganciclovir combination therapy (median; range)	Lamivudine-famciclovir combination therapy (median; range)	p-value
$\ln V_0$	19.43 (16.51-22.59)	18.76 (15.89-21.78)	21.39 (20.62-21.99)	0.78
c	1.07 (0.96-1.93)	1.34 (1.04-1.65)	1.62 (1.26-2.35)	0.60
ε	0.93 (0.89-0.98)	0.86 (0.80-0.91)	0.95 (0.94-0.96)	0.44
δ	0.08 (0.3-0.12)	0.19 (0.13-0.25)	0.10 (0.04-0.24)	0.50
T½ first phase ($\ln 2/c$)	16 hrs (9-17)	12 hrs (10-16)	10 hrs (7-13)	0.60
T½ second phase ($\ln 2/\delta$)	220 hrs (≈9 days) (144-628)	92 hrs (≈4 days) (66-126)	173 hrs (≈7 days) (70-397)	0.50

V_0 = viral load at start of therapy
c = turn-over rate of free virus
ε = effectiveness of antiviral compound in blocking viral replication
δ = death rate of infected hepatocytes
T½ = half-life

All patients finished the four-week treatment period and none of the patients experienced a serious adverse event. Most frequently observed adverse events are presented in table 3; they were comparable in the three groups.

Table 3 Most frequently observed side effects

	Lamivudine monotherapy	Lamivudine-ganciclovir combination therapy	Lamivudine-famciclovir combination therapy
Fatigue	1	2	1
Headache	1	0	2
Hey fever	1	0	1

Discussion

Lamivudine, a cytosine nucleoside analogue, is capable of interfering with the replication of the hepatitis B virus (HBV) by terminating either the negative or the positive proviral DNA-chain (15). Famciclovir and ganciclovir, guanine nucleoside analogues, cause proviral chain termination as well as inhibition of priming, the first step in viral polymerase-mediated replication (16-20). Simultaneous interference with different steps of viral replication could enhance the suppression of viral replication.

In this study, we describe a population of chronic hepatitis B patients with unfavorable baseline characteristics. First of all, the majority of patients had been treated with lamivudine previously. As we have shown in a previous dynamic study (21), re-treatment with lamivudine yields a less potent inhibition of viral replication in relation to that of lamivudine-naïve patients. Moreover, all patients had baseline serum transaminases which were only slightly elevated. In large studies on lamivudine-treated patients, baseline ALT has proven to be significantly related to HBeAg seroconversion (4, 5). Viral dynamic parameters were not significantly different between the three groups. If we look at the data more carefully, there seem to be two trends.

First of all, lamivudine-famciclovir combination therapy exhibited a stronger and more consistent pattern in blocking viral production than the other treatment arms. A previous study on viral dynamics in patients treated with lamivudine and lamivudine-famciclovir showed a significantly better effect of combination therapy as compared to lamivudine monotherapy (14). Some factors might be responsible for these differences. Although all patients in the study by Lau et al. were Asian -these patients often show less intrinsic activity against the HBV- mean serum transaminases were elevated in both groups. This is in contrast to our patients who all expressed only slightly elevated serum transaminases. In addition, the majority of patients in our study had been treated with lamivudine in the past; no data on previous therapy are mentioned in Lau's study. Lastly, modeling of viral decay is dependent on many assumptions. The bi-phasic model describes two phases of viral decay and therefore forces the HBV DNA levels to fit two slopes of viral decay. If the treatment period is longer, presumably the initial decline in viral replication will last longer and therefore greater effectiveness in blocking viral replication will be calculated.

The second striking result of our study is that the turnover of infected hepatocytes was much smaller in the lamivudine-ganciclovir group than in the other groups. An explanation for this observation remains speculative. Baseline ALT in this group, which could have an effect on the turnover of infected hepatocytes, was not significantly different from that in the other groups. Ganciclovir has a much stronger inhibitory effect on infected than on uninfected cells. But cells that express a high rate of DNA synthesis, such as hematopoietic progenitor cells, are more prone to interference and inhibition of ganciclovir (22). However, hepatocytes have a slow turn-over rate: it has been calculated that the half-life of infected hepatocytes is 10-100 days (23) and is even much longer for uninfected hepatocytes. Therefore, it does not seem very likely that ganciclovir has a direct cytotoxic effect on (un)infected hepatocytes.
Our study, which describes only a small population of patients, does not reveal a difference between the treatment arms. However, in vitro data, which were the basis for our study, suggest otherwise. The combination of lamivudine and famciclovir shows a synergistic effect in inhibiting viral replication (24-26). Active uptake into the hepatocyte as well as phosphorylation inside the hepatocyte depends on distinct mechanisms for lamivudine on the one hand and ganciclovir and famciclovir on the other hand (Fig 1) (24). However, deoxycitidine kinase, which catalyzes the conversion of lamivudine to lamivudine-monophosphate, is also capable of metabolizing other nucleoside analogue monophosphates (27). Moreover, one should realize that the situation as mimicked in cell culture systems cannot automatically be translated to the *in vivo* situation. These cell cultures provide homologous cell cultures and do not include the inter-subject diversity in metabolism, as can be observed in patients. Therefore, these studies in cell systems can only be used as an indicator of what to expect in *in vivo* situations.
This study in a small group of patients does not show a difference in viral dynamic parameters between the different treatment groups. It should be taken into account that both patient characteristics and modeling approaches influence the results, which are obtained in different studies. Because of the minimal additive antiviral effect of the combinations evaluated in this study over lamivudine monotherapy and the potential toxicity of ganciclovir in particular, we do not recommend either one of the combinations.

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SAFETY AND EFFICACY OF ORAL ENTECAVIR GIVEN FOR 28 DAYS IN PATIENTS WITH CHRONIC HEPATITIS B VIRUS INFECTION

R.A. de Man[1], L.M.M. Wolters [1], F. Nevens[2], D. Chua[3], M. Sherman[4], C.L. Lai[5], A. Gadano[6], Y.Lee[7], F.Mazzotta[8]. N.Thomas[9], D.DeHertogh[9]

[1]University Hospital Rotterdam, The Netherlands; [2]University Hospital Gasthuisberg, Leuven, Belgium; [3]Research Concepts, Chicago IL, USA; [4]Toronto General Hospital, Toronto, Canada; [5]Queen Mary Hospital, Hong-Kong, Hong Kong special; administrative region of China; [6]Hospital Italiano, Buenos Aires, Argentina; [7]NE Medical Center, Boston, USA; [8]S.M. Annunziata Hospital, Florence, Italy; [9]Bristol Myers Squibb, Wallingford CT, USA.

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Summary

Entecavir is an oral antiviral drug with selective activity against hepatitis B virus (HBV). We conducted a randomized, placebo-controlled, dose-escalating study in patients with chronic hepatitis B infection in which we evaluated the efficacy and safety of entecavir given for 28 days. Follow-up was 24 weeks.

All doses of entecavir (0.05 mg, 0.1 mg, 0.5 mg and 1.0 mg) showed a pronounced suppression of replication of the HBV with a 2.21, 2.29, 2.81 and 2.55 mean $\log_{10}$ reduction of viral load, respectively. Approximately 25% of patients on entecavir showed a decline of HBV DNA below the limit of detection of the Chiron HBV-DNA assay (<0.7 MEq/ml). In the post-dosing follow-up period patients who were treated with 0.5 and 1.0 mg of entecavir showed a considerably slower return in their HBV DNA levels to baseline compared with those patients treated with lower dosages ($p<0.05$). All doses of entecavir were well tolerated with no significant difference between treated patients and those receiving placebo. No significant changes in ALT levels within the dose groups and the placebo group between baseline and the end of treatment were observed. Three patients (9%) (one each in the 0.05, 0.1 and 0.5 mg group) experienced asymptomatic hepatitis flares 16 weeks (2 patients) and 24 weeks (1 patient) after withdrawal of entecavir.

In conclusion, in this 28-day study of entecavir a pronounced decrease of HBV DNA was observed and there were no significant side effects in entecavir patients in comparison with placebo-treated patients.

Introduction

Although the introduction of an effective vaccine against hepatitis B has drastically reduced the incidence of new infections, more than three hundred million people are affected by chronic hepatitis B infection world-wide. The infection may eventually lead to a substantial percentage of deaths due to cirrhosis with complications of liver failure and hepatocellular carcinoma (1). Alpha interferon has been the only registered therapy during recent years but it is effective in only one third of patients (2), requires parenteral administration and causes many side-effects especially in case of cirrhosis (3). Lamivudine, a cytosine nucleoside analogue, is an orally administered antiviral agent with few side-effects. It has recently been registered for the treatment of chronic hepatitis B infection (4-6). However, the development of mutations with decreased sensitivity of the virus for lamivudine (7-9), and the rebound of viral replication after withdrawal of the drug (10, 11) leave room for further improvement of nucleoside analogue therapy. The increase in viral replication after withdrawal of the drug is based on residual cccDNA inside the nucleus of the hepatocyte which is not affected by lamivudine (12, 13).
Entecavir, a new deoxyguanine nucleoside analogue, is a selective inhibitor of the replication of the hepatitis B virus (14-16). In HepG2.2.15 cell lines, this compound has proved to be 30 times more potent than lamivudine in suppressing viral replication with an EC_{50} of 4 nM compared to 116 nM for lamivudine (14). The *in vitro* therapeutic index (a marker indicating the range of doses which can be applied safely without causing toxicity) is 8000 (15). In addition, incorporation of entecavir into cellular polymerases appears to be very inefficient thus bypassing an important cause of *in vivo* toxicity of nucleoside analogues. In chronically infected woodchucks, up to 8 $\log_{10}$ reduction in viral DNA has been observed after a mean of 32 weeks of therapy. Ten woodchucks which were treated for at least 14 months were both negative for cccDNA and HBcAg in the liver biopsy (17, 18). Five animals that were kept on maintenance therapy for up to three years showed sustained drops in woodchuck hepatitis virus (WHV) DNA with no evidence of resistance. In addition, WHV DNA remained undetectable for 21 months after withdrawal of the drug (19). This may indicate that entecavir is not only capable of interfering with viral replication, but that it also has a direct effect on cccDNA. In untreated historical controls, all chronically infected woodchucks die of hepatocellular carcinoma within four years. In contrast, entecavir reduces the incidence of liver cancer resulting in prolonged survival of the animals.
To be able to explore the initial antiviral effect and safety of entecavir in chronic hepatitis B patients, a placebo-controlled, dose-escalating study was performed.

Patients and methods

Study design
We conducted a double blind, placebo-controlled dose-escalating study of four dosages of entecavir 0.05 mg, 0.1 mg, 0.5 mg and 1.0 mg once daily. If proved eligible during the two screening visits, patients were treated for 4 weeks and followed up for 24 weeks after discontinuation of the drug. Each cohort started when the evaluation of the previous cohort treated with a lower dosage had proved to be safe. In each cohort, patients were randomly assigned to a dose of entecavir or placebo (8:2). Blinded randomisation was performed using a predetermined schedule maintained by the sponsor; bottle assignments were communicated by phone to the individual investigator. HBV DNA was assessed at 2 screening visits, day 1 (baseline), week 1, 2, 3 and 4 during dosing and week 2 and 4 post-dosing. HBV serology was obtained during initial screening, day 1, week 4 and final post-dosing visit. Safety analysis, based on laboratory results, clinical evaluation of adverse events and physical

examination, was assessed on a weekly basis during therapy and monthly during follow-up. The protocol was approved by the Medical Ethics Committee of each participating center. All patients had to give written informed consent.

Selection of patients
Eligible patients included men and woman older than 16 years. Chronic hepatitis B infection was documented by HBsAg positivity in the serum for over 24 weeks before start of therapy. Each individual had to have an HBV DNA >20MEq/ml by the bDNA assay on 2 determinations at least two weeks apart. Patients had to have a compensated liver disease as documented by serum transaminase activity below 5 times the upper limit of normal (ULN), serum albumin >30 g/l, serum bilirubin < 51.3 µmol/l, a prothrombin time which was not elongated for more than 3 seconds and the absence of significant ascites, hepatic encephalopathy or variceal bleeding. Both HBeAg positive and HBeAg negative patients were eligible. Previous antiviral therapy, such as alpha interferon and other nucleoside analogues, was permitted but had to be withdrawn 6 months before start of therapy in this trial. Patients were excluded if they were co-infected with the hepatitis C virus, the hepatitis D virus or the Human Immunodeficiency Virus (HIV); had another concomitant liver disease; had any signs or a history of pancreatitis; had received immunosuppressive therapy within six months before start of therapy. Both male and female patients had to practice a reliable method of contraception.

Assays
HBsAg, antibodies to HBsAg, HBeAg, antibodies to HBeAg, anti-HCV, anti-HDV and anti-HIV were analyzed by an enzyme immunoassay (Abbott Diagnostics,Abbott Chicago). If the anti-HIV assay proved to be positive, an HIV Western blot (Biorad, Hercules, CA) was performed for confirmation. HBV DNA was detected with the Chiron branched DNA signal amplification assay (bDNA, Chiron, Emeryville, CA;) lower limit of detection of $0.7x\ 10^6$ genome equivalents/ml (geq/ml). HBV DNA was assessed with a sensitive PCR assay (Roche Monitor, limit of detection 400 geq/ml; Roche, Indianapolis, IN) at baseline and on day 28.

Statistics
The primary endpoint of this study was the proportion of subjects who had either a ≥ 2 $\log_{10}$ reduction from baseline in their day 28 bDNA assay or who achieved undetectable levels of HBV DNA by the bDNA assay and a ≥ 2 $\log_{10}$ reduction from baseline in their day 28 Roche HBV DNA PCR assay. The secondary efficacy summaries were the proportion undetectable by the bDNA assay, and the mean $\log_{10}$ reduction from baseline by the bDNA assay regarded as a continuous parameter at days 7, 14, 21, 28 and post-dosing days 14 and 28.
For comparison between entecavir and placebo for endpoints represented by binary variables used Fisher's exact test. Comparisons of HBV DNA mean reductions were based on t-tests.
One subject receiving entecavir 0.1 mg had a large spontaneous reduction in HBV DNA levels between the screening and baseline visits and was excluded from all of the efficacy analyses. The placebo subjects from each cohort in the sequential designs were pooled for comparison with each entecavir dose group. For binary end points, subjects who discontinued before day 28 because of an adverse event were assigned the failure value. Estimates and comparisons of mean differences were based on all available measurements at each time point, with measurements made after a subject who prematurely discontinued dosing was excluded.

Results

A total of forty-two patients were randomized into this study. Initially, the protocol was scheduled to include doses of 0.1 mg (cohort 1), 0.5 mg (cohort 2), 1.0 mg (cohort 3) and 2.5 mg (cohort 4) of entecavir. However, after virologic evaluation of the 0.1 mg and 0.5 mg dosages of entecavir, it was decided to amend the protocol to exclude the highest dose of 2.5 mg and instead to include a lower dose of 0.05 mg (cohort 5). Eight, 9, 9, and 8 patients were treated with 0.05 mg, 0.1 mg, 0.5 mg, 1.0 mg, respectively. The evaluation was completed with a placebo group of 8 patients in which all data of placebo treated patients per cohort were pooled. Baseline characteristics are shown in Table 1. All entecavir groups and the placebo group were comparable with regard to sex and age. Fifty-two percent of patients were of Asian origin (n=22). Approximately fifty percent of patients were treatment-naïve at start of entecavir therapy. In the two lower dose entecavir groups, half of the patients were previously treated with lamivudine. The majority of patients were HBeAg positive (n=38, 90%). The baseline level of HBV DNA was comparable in all cohorts. Mean ALT level was above the Upper Limit of Normal (ULN) in all dosage groups.

Table 1 Baseline demographics/characteristics, virology and chemistry

	Entecavir 0.05 mg n=8	Entecavir 0.1 mg n=9	Entecavir 0.5 mg n=9	Entecavir 1.0 mg n=8	Placebo n=8
Sex (M:F)	6:2 (75%M)	8:1 (89%M)	7:2 (78%M)	8:0 (100%M)	7:1 (88%M)
Age (Mean years)	33.8	45.1	35.2	41.1	42.1
Race					
Asian	5 (63%)	6(67%)	3(33%)	2 (25%)	6 (75%)
Caucasian	2 (25%)	3 (33%)	4 (44%)	3 (38%)	2 (25%)
Black	0	0	1 (11%)	0	0
Hispanic/ Latino	0	0	0	3 (38%)	0
Other	1 (13%)	0	1 (11%)	0	0
Prior HBV therapy	4 (50%)	5 (56%)	4 (44%)	3 (38%)	2 (25%)
IFN only	0	1 (11%)	4 (44%)	1 (13%)	1 (13%)
Lamivudine only	0	1 (11%)	0	0	0
Lamivudine+IFN	4 (50%)	2 (22%)	0	2 (25%)	1 (13%)
Lamivudine+ polyclonal antibodies	0	1 (11%)	0	0	0
HBeAg positivity	7 (88%)	8 (89%)	7 (78%)	8 (100%)	8 (100%)
Mean (log_{10} HBV bDNA)	2.7	3.0	3.2	3.1	3.0
ALT (IU/l, standard deviation)	77 (52)	78 (54)	106 (74)	124 (79)	86 (65)

Virological response

All entecavir-treated patients showed a pronounced decline of serum HBV DNA after 4 weeks of therapy. Treatment with 0.05 mg, 0.1 mg, 0.5 mg and 1.0 mg of entecavir resulted in a 2.21, 2.29, 2.81 and 2.55 mean $\log_{10}$ decline respectively versus 0.012 $\log_{10}$ increase in the placebo group (Table 2, Figure 1). Reduction in HBV DNA was similar between pre-treated patients and treatment-naive patients. HBV DNA below the limit of quantification of the bDNA assay was observed in 25% of patients in the two lowest dosage groups (0.05 and 0.1 mg), in 33% of the patients treated with 0.5 mg of entecavir and in 13% of patients treated with 1.0 mg of entecavir. HBV DNA levels in the first 4 weeks post-dosing remained significantly lower for the two higher dosages of entecavir (0.5 and 1.0 mg) than for the 0.05 or 0.1 mg dosages (p=0.005). Three entecavir-treated patients had undetectable HBV DNA by the bDNA assay 6 months post-dosing (2 at 0.5 mg and 1 at 0.1 mg). Two patients, 1 treated with 0.1 mg and 1 with 0.5 mg, lost HBeAg transiently. In these patients HBeAg was undetectable at day 28 and 4 weeks post-dosing, but again detectable at 6 months post-dosing. A third subject in the 0.05 mg group had lost HBeAg at 6 months post-dosing.

Table 2 Response during 1 month of entecavir

	Entecavir 0.05 mg n=8	Entecavir 0.1 mg n=8[a]	Entecavir 0.5 mg N=9	Entecavir 1.0 mg n=8[a]	Placebo n=8
Mean $\log_{10}$ change in HBV DNA	-2.21 (0.10)	-2.29 (0.33)	-2.81 (0.21)	-2.55 (0.17)	+0.01 (0.13)
Patients reaching the primary endpoint[b]	7 (88%)	4 (50%)	8 (89%)	6 (75%)	0
p-value for comparison to placebo	0.0001	0.077	0.0004	0.007	
Percentage undetectable by the bDNA assay	2 (25%)	2 (25%)	3 (33%)	1 (13%)	0

[a] subjects discontinuing dosing before week 4 are excluded from the mean $\log_{10}$ change and are assigned failure for the analysis of the primary endpoint.

[b] $\geq 2\log_{10}$ reduction in HBV DNA by bDNA or undetectable HBV DNA by bDNA and $\geq\log_{10}$ reduction by Roche PCR.Level of detection of bDNA was 0.7×10^6 Eq/ml.

Liver transaminase levels

Mean transaminase levels were elevated in all dosage groups at start of therapy (Table 1). Both at baseline and at the end of therapy, no significant difference was observed between the four dosage groups and placebo group. Moreover, no significant difference between baseline and end of therapy levels of ALT were observed within the treatment groups.

In two patients both in the 0.1 mg group ALT levels rose during the dosing period. Both patients were clinically asymptomatic while bilirubin stayed normal. Patient 1 entered the study with ALT level of 73 IU/l, at the end of the treatment period ALT was 165 IU/l .Patient 2 entered the study with ALT level of 173 IU/l which increased to 452 IU/l, entecavir was discontinued with a subsequent drop in ALT. Patient 1 had a 2.8 log reduction in HBV-DNA and patient 2 had 3.3 log reduction in HBV-DNA at study drug discontinuation mandated by the protocol.

Elevation of serum transaminases during the post-treatment follow-up are presented in Table 3. During follow-up, three patients experienced a hepatitis flare with ALT levels which were more than 2 times baseline and greater than 10 times the upper limit of normal (ULN). None of these were associated with increase in bilirubin. In two patients (one patient treated with 0.05 mg entecavir and one patient treated with 0.1 mg) the flare was observed at 16 weeks post dosing. Serum ALT levels had declined to 5.7xULN and 2.2xULN respectively at the last follow-up visit (24 weeks post-dosing). In the third patient, who had been treated with 0.5 mg of entecavir, the flare was observed at 24 weeks post-dosing. No further follow-up data on this patient are available.

Table 3 Elevation of serum transaminases in the post-treatment period of the study

	Entecavir 0.05 mg n=8	Entecavir 0.1 mg n=9	Entecavir 0.5 mg N=9	Entecavir 1.0 mg n=8	Placebo n=8
ALT					
>2x baseline value	3 (38%)	0	3 (33%)	2 (25%)	1 (13%)
>3x baseline value	3 (38%)	1 (11%)	2 (22%)	1 (13%)	0
>10x ULN[a]	1 (13%)	1 (13%)	1 (13%)	0	0
AST					
>2x baseline value	2 (25%)	1 (11%)	3 (33%)	1 (13%)	1 (13%)
>3x baseline value	2 (25%)	1 (11%)	2 (22%)	1 (13%)	0
>10x ULN[a]	1 (13%)	0	0	0	0

[a] Upper Limit of Normal

Safety

Overall, entecavir was well tolerated and no dose-related or dose-limiting toxicity occurred. The majority of patients reported adverse events which were of mild to moderate severity but not significantly different from placebo-treated patients. Fatigue and headache were reported most frequently (Table 4).One subject in the 0.5 mg entecavir group experienced asymptomatic grade 1 elevation of amylase and a grade 3 elevation of serum lipase on day 8 of therapy. This patient was known to have had elevation of lipase in the past. The study drug was withdrawn for several days during which time both enzymes returned to normal and study drug was reintroduced and completed without further problems.

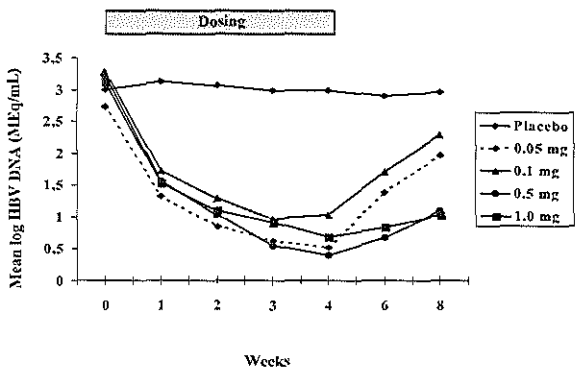
During the dosing and post-dosing period, a total of 5 serious adverse events were reported, most of which could be related to progression of liver disease or external interference. One occurred in the first cohort (0.1 mg).This patient was diagnosed with hepatocellular carcinoma and pulmonary fibrosis six months post-dosing. In the second cohort (0.5 mg), one patient was hospitalized for a cholecystectomy for biliary stones three months after withdrawal of therapy and another patient experienced septic shock after a heroin overdose 1 month post-dosing. In the third cohort (1.0 mg), one patient was withdrawn from therapy after 6 days as specified in the protocol due to persistent headache lasting longer than 12 hours. Extensive neurologic evaluation did not show any abnormalities. The other patient was involved in a car accident which required short-term hospitalization 2 months after withdrawal of therapy. No adverse events have been reported in the fifth cohort, the lowest dose applied.

Table 4 Most common non-serious adverse events reported on study[a].

	Entecavir 0.05 mg n=8	Entecavir 0.1 mg n=9	Entecavir 0.5 mg n=9	Entecavir 1.0 mg n=8	Placebo n=8
Fatigue	2 (25%)	4 (44%)	2 (22%)	4 (50%)	3 (38%)
Headache	2 (25%)	3 (33%)	4 (44%)	3 (38%)	3 (38%)
Infection	2 (25%)	0	1 (11%)	3 (38%)	0
Abdominal pain	1 (13%)	3 (33%)	1 (11%)	3 (38%)	2 (25%)
Flu syndrome	1 (13%)	0	2 (22%)	4 (50%)	3(38%)
Pharyngitis	1 (13%)	3 (33%)	1 (11%)	0	2 (25%)
Nausea	0	2 (22%)	1 (11%)	3 (38%)	0

[a] Includes events reported during 4-week dosing period plus 24-week post-dosing follow-up.

Fig 1 Mean HBV DNA during therapy and 1 month follow-up.



Discussion

The present study reports experience with the new nucleoside analogue, entecavir, in patients with chronic hepatitis B. All dosages of entecavir showed a pronounced decline of HBV DNA of more than two log after 4 weeks. In some patients, HBV DNA declined below the limit of detection ($0.7x10^6$ Eq/ml) of the bDNA assay. Both interferon and lamivudine pre-treated patients and treatment-naïve patients responded well to therapy. A transient loss of HBeAg occurred in two patients. One month of treatment is limited and not capable of inducing a pronounced viral suppression coinciding with HBeAg seroconversion. After withdrawal of entecavir in the 0.5 and 1.0 mg dose groups, HBV DNA remained significantly below baseline levels for four weeks. These data are in agreement with results from *in vitro* studies of entecavir and data on inhibition of viral replication in woodchucks (16,17).Rebound of virus after withdrawal of lamivudine occurs quickly. Return of HBV DNA to baseline was observed within 1 month of cessation of lamivudine in a phase II 24-week dosing study for the treatment of chronic hepatitis B patients (5). A comparison of studies in which patients were treated with lamivudine for 3, 6 and 12 months (4-6) showed that 12 months of therapy resulted in a more gradual rebound of viral load after discontinuation of therapy. However,

the latter study did not report on the extent of decline of HBV DNA with more sensitive assays. A larger percentage of patients with undetectable HBV DNA by PCR, may cause a slower return to baseline. In our study of entecavir, most of the patients in all dosage groups still had detectable HBV DNA by the bDNA assay at the end of 4 weeks of therapy although the higher dosage groups showed a more gradual return to baseline in the post-dosing period.
As has been observed after withdrawal of lamivudine therapy (20), withdrawal of entecavir with a subsequent return of viral replication may induce a flare of serum transaminases. Spontaneous hepatitis flares occur at an annual rate of 27% in HBeAg positive patients (21) and larger patient populations should be evaluated to explore the relationship between entecavir therapy and the occurrence of hepatitis flares. None of the patients in the placebo group in our study population experienced a flare of serum transaminases, whereas 9% of patients who were treated with entecavir experienced transient hepatitis flare 16-24 weeks after withdrawal of entecavir. More pronounced suppression of HBV DNA may lead to a delay in recurrence in viral replication as well as a delay of the hepatitis flare.
Nucleoside analogues are capable of interfering with the replication of the HBV since they have the same characteristics as natural nucleosides. However, this may also lead to interference with cellular polymerases. As has been seen with some other nucleoside analogues, inhibition of cellular enzymes may cause significant clinical events and even deaths (22-25). Entecavir has proved to have very low affinity for cellular DNA. In particular, mitochondrial DNA does not use entecavir at all. In this human study, no major adverse events were detected that could have been related to this characteristic of nucleoside analogues, but long-term studies will be needed to clarify this further.
In conclusion, entecavir can be given safely for a short period of time and causes pronounced reduction in HBV DNA levels with a slower rebound after stopping therapy than has been reported for lamivudine. Entecavir should be studied in longer-term dosing trials to be able to evaluate more definitively its effect on viral replication and cccDNA, and ultimately cure of chronic hepatitis B infection.

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8

VIRAL DYNAMICS DURING AND AFTER ENTECAVIR THERAPY IN PATIENTS WITH CHRONIC HEPATITIS B

L.M.M. Wolters[1], B.E. Hansen[2], H.G.M. Niesters[3], D. DeHertogh[4], R.A. de Man[1]

Departments of [1]Gastroenterology & Hepatology, [2]Epidemiology & Biostatistics, [3]Virology, University Hospital Rotterdam, The Netherlands, [4]Bristol-Myers Squibb, Wallingford, CT, USA

Submitted

Summary

Introduction Nucleoside analogues inhibit hepatitis B virus (HBV) replication. Entecavir, a new guanine nucleoside, has also shown to reduce cccDNA to undetectable levels in woodchucks chronically infected with hepatitis virus. Mathematical description of changes in viral load during and after therapy may help to understand the several events that take place during nucleoside analogue treatment.
Patients and methods Ten chronic hepatitis B patients were evaluated with a mathematical model during and after withdrawal of four doses of entecavir. Blood was drawn for HBV DNA measurement at frequent intervals. Non-linear modeling was used to fit individual patient data.
Results The median effectiveness in blocking viral production is 96% (n=10, range 87%-98%). The median half-life of viral turn-over was 16 hours (range 12-29). The median half-life of infected hepatocytes was 257 hours (=10.7 days) (n=9, range 112-762). Rebound of viral replication also followed a bi-phasic return to baseline levels.
Conclusion Decay and rebound of viral concentration during and after entecavir therapy respectively, showed a bi-phasic pattern. Both can be described with a mathematical model. Data on levels of cccDNA in the liver in these patients could be helpful in supporting the parameters as calculated with the model.

Introduction

In patients with chronic hepatitis B infection, annual clearance of HBsAg and HBeAg is estimated at 1% and 10% respectively. HBeAg clearance, which is immune mediated, is improved by alpha interferon therapy resulting in HBeAg seroconversion in 30-40% of patients (1-4). Especially those patients with an immune-tolerant status, a large part of which is originating from Asian countries, do not show a favourable response to alpha interferon therapy (5). Recently, lamivudine has been registered as a second option for the treatment of chronic hepatitis B patients. Whereas HBeAg seroconversion is a solid end-point for alpha interferon therapy, durability of HBeAg seroconversion after withdrawal of lamivudine therapy needs to be evaluated yet. Reports on this end-point are contradictory (6-8). Recurrence of viral activity is attributed to the remnant covalently closed circular DNA (cccDNA) inside the nucleus of hepatocytes which is not affected by lamivudine (9-10).
Entecavir, a new guanine nucleoside analogue which is currently under investigation in phase II studies, is believed to be capable to interfere with cccDNA (11-13). This consideration is based on observations in woodchucks chronically infected with the woodchuck hepatitis B virus. Short-term entecavir therapy markedly reduces cccDNA levels in the liver of woodchucks (14) and rebound of virus after withdrawal of therapy in woodchucks (15). Moreover, maintenance therapy in woodchucks with once weekly dosing regimens, is able to reduce cccDNA in the liver to undetectable levels (16).
Mathematical modeling can be used to evaluate the mechanism of action of entecavir on both viral decline during and the return of virus after withdrawal of therapy. In previous modeling studies on the effect of nucleoside analogues in a chronic hepatitis B infection, it has been shown that viral decline can be divided into two phases: a first phase of turnover of free virus and a second phase of death of infected hepatocytes (17,18). Return of virus after withdrawal of therapy has never been evaluated in detail yet, but may be helpful in clarifying the mechanisms that take place during viral replication.
We therefore conducted a study to model viral decline during entecavir therapy and viral return after withdrawal of entecavir therapy.

Patients and Methods

Study design
All patients who were treated in the Academic Hospital Rotterdam, the Netherlands, in a study on the safety and efficacy of entecavir, were recruited for a study on viral dynamics. Patients were treated in a one month, double-blind, placebo-controlled dose escalating study on the safety and efficacy of entecavir (0.05 mg, 0.1 mg, 0.5 mg, 1.0 mg) versus placebo with a follow-up of six months.
During the first month of therapy, HBV DNA was measured at day 1 at t=0 and 8 hours, at day 2 at t=24 and 32 hours and at day 3, 4, 7, 10, 14, 21 and 28. Follow-up after withdrawal of therapy was documented with HBV DNA measurements at day 29, 30, 31, 32, 35, 38, 42, 49, 56 and month 3, 4, 5 and 6.

Selection of patients
Patients were screened for eligibility on two occasions which had to be at least two weeks apart. Eligible patients included men and woman older than 18 years with a chronic hepatitis B infection as documented by HBsAg positivity in the serum for over 24 weeks before start of therapy and HBV DNA > 20 Meq/ml measured with the Chiron hybridization bDNA assay. Patients had to have a compensated liver disease as documented by laboratory and clinical

evaluation. Both HBeAg positive and HBeAg negative patients could be included. Previous antiviral therapy with alpha interferon, other nucleoside analogues or immunosuppressive therapy was permitted but these drugs had to be withdrawn 6 months before start of therapy in this trial. Patients were excluded if they were co-infected with the hepatitis C virus, the hepatitis D virus or the Human Immunodeficiency Virus; had another concomitant liver disease; had a history of a pancreatitis; had a history of any form of chronic headaches. Both male and female patients had to practice a reliable method of contraception.

Assays

HBV DNA was quantified with a Digene Hybrid Capture tube liquid hybridisation assay (calibrated on the EUROHEP standard (19)). If HBV DNA declined below $1.5x10^6$ geq/ml (the limit of detection of this liquid hybridisation assay) during therapy, it was reassessed with the quantitative PCR (Roche, Amplicor Diagnostics, Almere, The Netherlands calibrated on the EUROHEP standard; lower limit of detection of 1000 geq/ml). HBV polymerase mutant analysis was performed with the INNO-LiPA strip (Innogenetics, Ghent, Belgium) (20).

Modeling of viral decline and rebound

A bi-phasic model previously applied for viral decline in chronic hepatitis C patients during alpha interferon therapy, was used to describe viral decay, by means of viral dynamic parameters, during entecavir therapy (21). In short, viral decline in this model is described by the following equation:

$\ln V(t)=\ln V_0+\ln\{A\exp[-\lambda_1 t]+(1-A)\exp[-\lambda_2 t]\}$, which equals

$V(t)= V_0\{A\exp[-\lambda_1 t]+(1-A)\exp[-\lambda_2 t]\}$ where

- λ_1 = slope of the first phase of viral decline
- λ_2 = slope of the second phase of viral decline
- A = $(\varepsilon c-\lambda_2)/(\lambda_1-\lambda_2)$
- $\lambda_{1,2}$ = $\frac{1}{2}\{(c+\delta)\pm[(c-\delta)^2+4(1-\varepsilon)(1-\eta)c\delta]^{1/2}\}$
- V_0 = initial viral load
- t = time
- δ = death rate of productively infected cells
- c = clearance rate of the free virus
- ε = effectiveness of entecavir in blocking virion production from infected cells
- η = effectiveness of entecavir in blocking de novo infection of susceptible cells

The bi-phasic return of virus after withdrawal of therapy was described by adapting a similar bi-phasic model as an inverse image of the bi-phasic decline in viral load during antiviral therapy:

$\ln V(t)^{\#}= \ln V_0^{\#}-\ln\{A^{\#}\exp[-\lambda_1^{\#}t^{\#}]+(1-A^{\#})\exp[-\lambda_2^{\#}t^{\#}]\}$, which equals

$V(t)^{\#}= V_0^{\#} / \{A^{\#}\exp[-\lambda_1^{\#}t^{\#}]+(1-A^{\#})\exp[-\lambda_2^{\#}t^{\#}]\}$ where

- $V_0^{\#}$ = viral load at the moment of withdrawal of therapy
- $t^{\#}$ = time after withdrawal of therapy
- $A^{\#}$ = multiplier
- $\lambda_1^{\#}$ = slope of the first phase of viral rebound
- $\lambda_2^{\#}$ = slope of the second phase of viral rebound

Statistics

Patients were fitted individually. Due to the small sample size, all patients on entecavir therapy were evaluated as one group. Non-linear modeling was used to fit both the bi-phasic model and the inverse bi-phasic model, executed in the PROCNLIN in SAS 6.12.

The Mann-Whitney test was used to compare the difference between dose groups in rebound of viral replication after withdrawal of entecavir. The Kruskal-Wallis test was applied to calculate the difference in dose of entecavir with regard to parameters of viral return. Significant difference was achieved if $p<0.05$.

Results

Eleven patients participated in the study: three patients received 0.05 mg, two 0.1 mg, two 0.5 mg, three 1.0 mg and one placebo. Ten patients on entecavir therapy were evaluated for viral dynamic parameters of viral decay. Nine patients were evaluated for re-appearance of viral replication after withdrawal from therapy; one patient was withdrawn from therapy after 1 week because of a serious adverse event and not included in the analysis for return of viral replication.

Table 1 Baseline characteristics

	Entecavir 0.05 mg n=3	Entecavir 0.1 mg n=3	Entecavir 0.5 mg n=2	Entecavir 1.0 mg n=2
Sex (M:F)	1:2	2:1	1:1	2:0
Age (Years; range)	23 (20-63)	39 (29-51)	38 (18-58)	27 (19-35)
Race				
Asian	0	2	0	1
Caucasian	1	1	1	1
Other	2	0	1	0
Previous lamivudine therapy	3	3	0	1
HBeAg positivity	3	3	2	2
HBV DNA (geq/ml) (median, range)	$4{,}15\times10^{9}$	$6{,}24\times10^{8}$	$8{,}34\times10^{9}$	$1{,}70\times10^{9}$
ALT xULN (median, range)	1 (1-1.5)	1.2 (1-3.4)	1.6 (1-2.2)	1.3 (1-1.5)

Baseline characteristics are shown in table 1. Six patients were male and 4 were female. The median age of the entecavir treated population was 35 years (range 18-63). Three patients were Asian and 4 patients Caucasian. The majority of patients (70%) were treated with lamivudine previously. Patient 4 and patient 10 had detectable mutant virus against lamivudine at start of entecavir therapy (YIDD and YVDD respectively). All patients were positive for HBeAg at start of therapy. Median baseline HBV DNA was 1.68×10^{9} geq/ml (range 5.52×10^{7}-1.50×10^{10}), median elevation of ALT at baseline was 1.1 x the Upper Limit of Normal (ULN) (range 1-3.5).

Viral decay was determined during 28 days of entecavir therapy (Figure 1). The median effectiveness of blocking viral replication in all ten patients on entecavir therapy was 96% (range 87-98%). Turn-over of free virus was 16 hours (median; range 12-29 hours, n=10), turn-over of infected hepatocytes was estimated to be 10.7 days (range 5.2-31.8 days, n=9). For calculation of the viral decline during the second phase, patient 10 was excluded. This patient discontinued medication after 1 week due to a serious adverse event (Table 2). Entecavir was still capable of blocking viral replication in both patients with detectable lamivudine induced mutant virus (effectiveness in blocking viral production of 87% and 98% respectively).

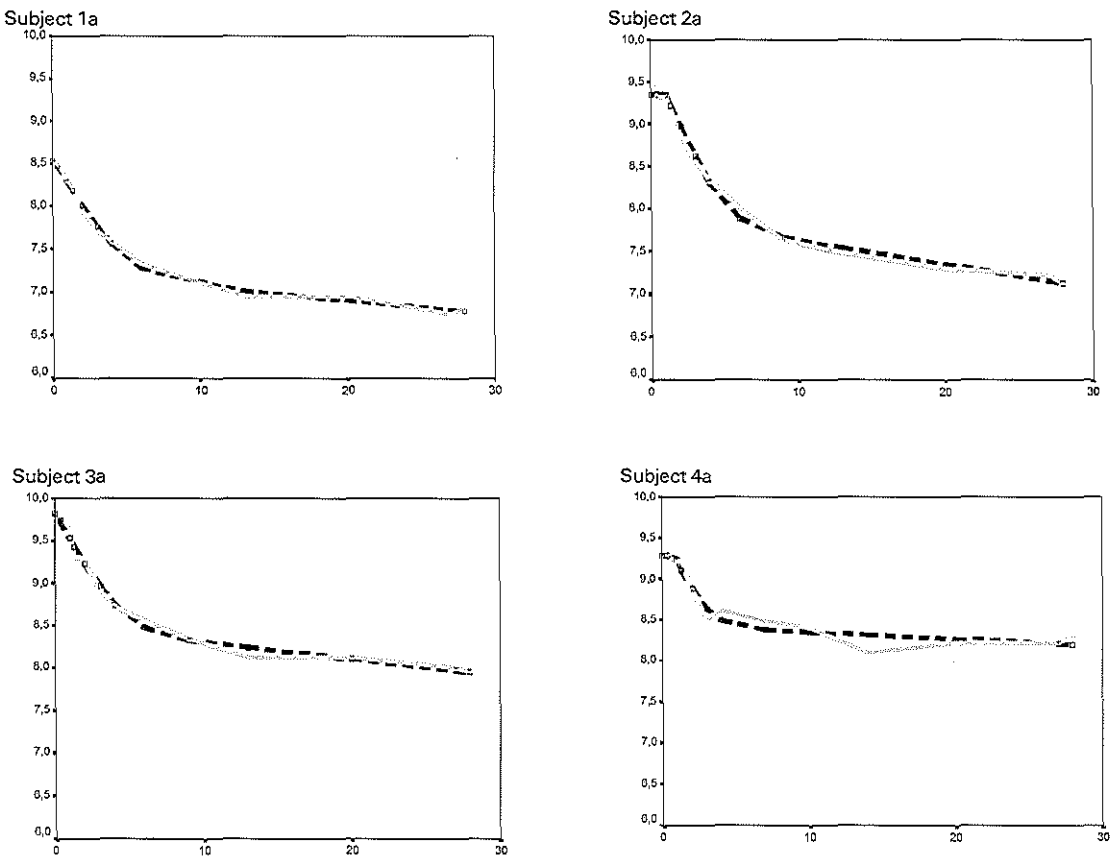
Table 2 Dynamic parameters during and after one month of entecavir therapy.

	During entecavir therapy				After withdrawal of entecavir therapy		
Patient	Effectiveness in blocking viral production	Half-life first phase	Half-life second phase	Initial viral load (geq/ml)	Doubling time of virus	Duration of fast increase of viral concentration (first phase of viral return)	Viral load at the end of follow-up
1; 0.05 mg	96%	24 hours	18.6 days	$3.40x10^8$	95 hours	23 days	$2.58x10^9$
2; 0.05 mg	97%	18 hours	10.7 days	$2.25x10^9$	129 hours	40 days	$1.83x10^9$
3; 0.05 mg	96%	22 hours	15.2 days	$6.63x10^9$	120 hours	30 days	$4.21x10^9$
4; 0.1 mg	87%	16 hours	31.8 days	$1.92x10^9$	140 hours	16 days	$1.05x10^9$
5; 0.1 mg	96%	13 hours	5.2 days	$5.18x10^8$	62 hours	14 days	$2.32x10^7$
6; 0.1 mg	89%	29 hours	25.0 days	$7.03x10^8$	102 hours	12 days	$3.06x10^8$
7; 0.5 mg	97%	12 hours	4.7 days	$1.44x10^9$	247 hours	101 days	$2.24x10^9$
8; 0.5 mg	95%	13 hours	5.6 days	$8.16x10^9$	244 hours	89 days	$5.35x10^9$
9; 1.0 mg	92%	16 hours	10.7 days	$3.61x10^7$	230 hours	109 days	$8.33x10^8$
10; 1.0 mg	98%	17 hours	N.A.*	$3.04x10^9$	N.A.*	N.A.*	N.A.*
Median	96%	16 hours	10.7 days	$1.92x10^9$	129 hours (≈5 days)	30 days	$1.05x10^9$

*Not applicable; this patient was withdrawn from therapy after 1 week.

Rebound of viral replication was followed until 6 months after withdrawal of therapy (n=9, excluding patient 10) (Table 2) (Figure 1). For mathematical description of return of viral concentration, the inverse of the bi-phasic model for viral decay describes the observed patient data accurately. The doubling time was 129 hours (median, range 62-247 hours) and the slope of the second phase of the rebound in viral concentration approaches zero in all patients (median 0.0304,range –0.000166 –0.00158). The change from the first to the second phase of viral return was calculated to be at a median of 30 days (range 12-109). No relation between viral load at the moment of withdrawal of entecavir, first and second phase of viral rebound with the dose of entecavir could be found. However, the 3 patients in the higher dose groups, showed a more gradual return of viral replication to baseline levels, than did the 6 patients in the lower dose groups (p=0.024).

Fig 1 Panel A: Viral decline during one month of entecavir therapy in 10 patients.
X-axis: 0-30 days Y-axis: level of HBV DNA on a log scale
Straight grey line: observed HBV DNA data
Black dotted line: fitted HBV DNA data



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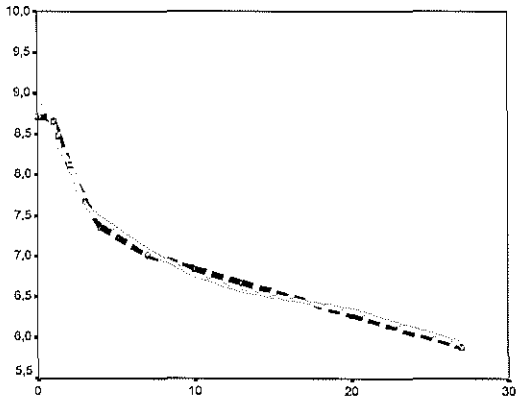

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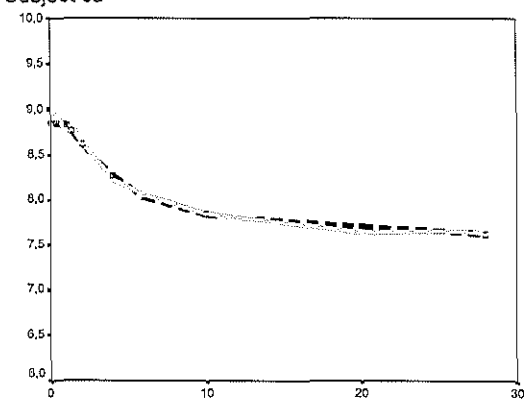

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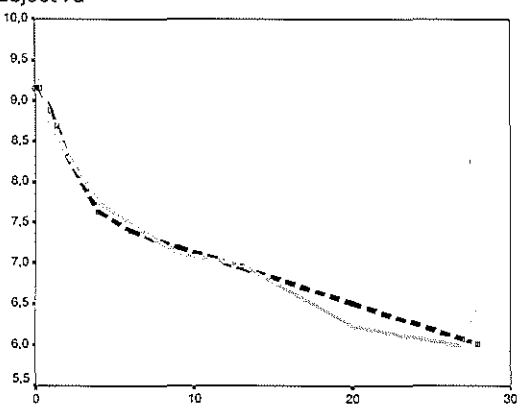

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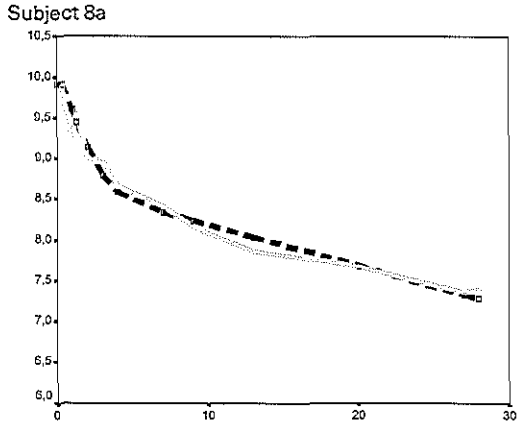

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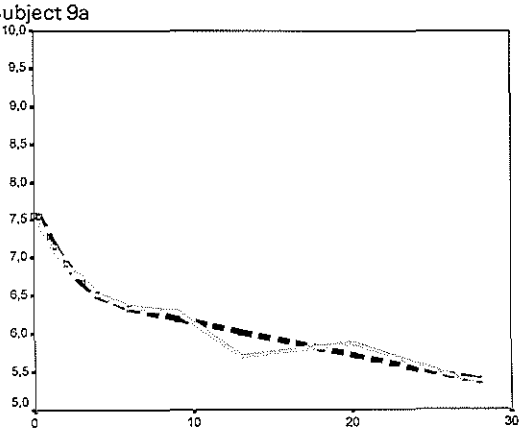

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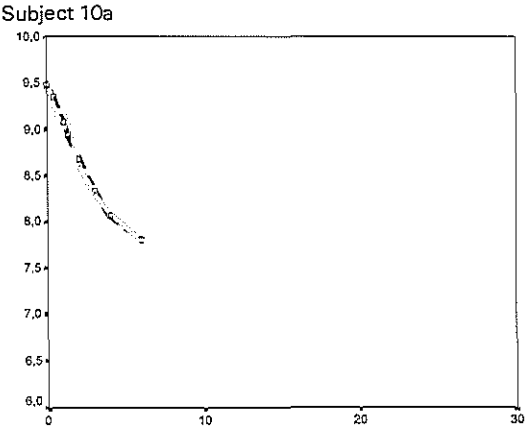
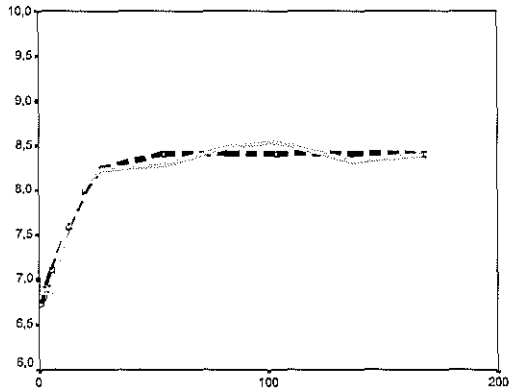


Fig 1 Panel B: Viral rebound in 9 patients during six months after withdrawal of entecavir
X-axis: 0-200 days Y-axis: level of HBV DNA on a log scale
Straight grey line: observed HBV DNA data
Black dotted line: fitted HBV DNA data

Subject 1b



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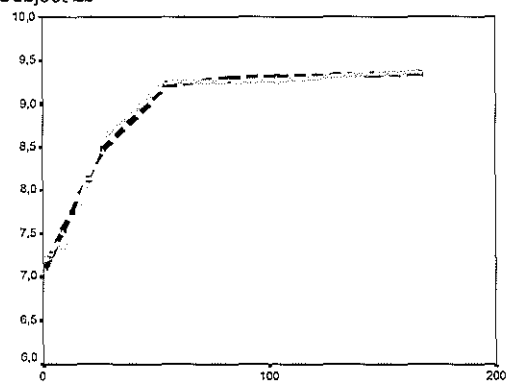

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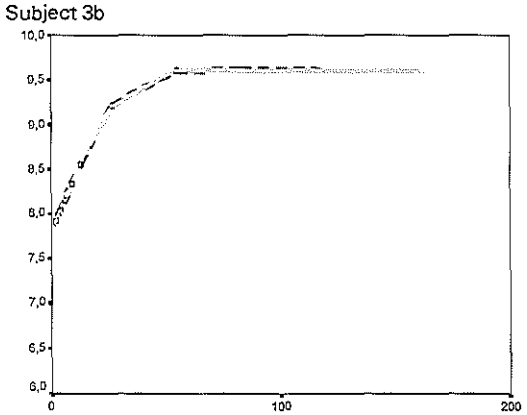

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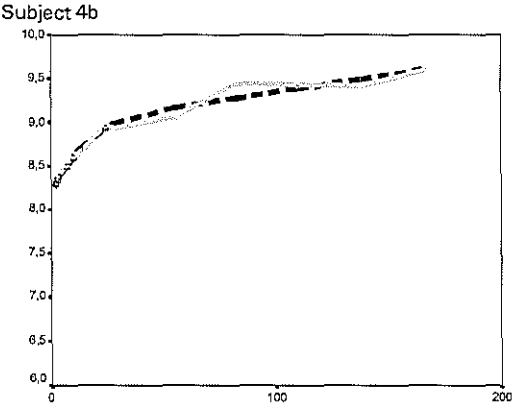

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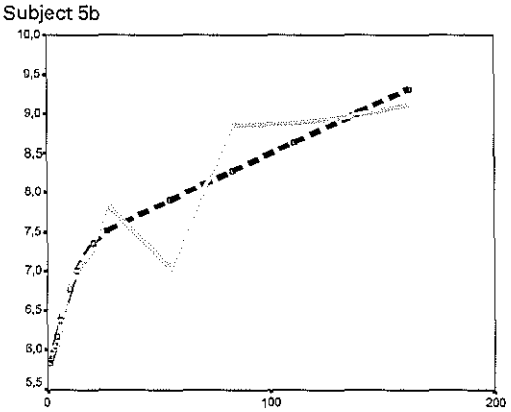

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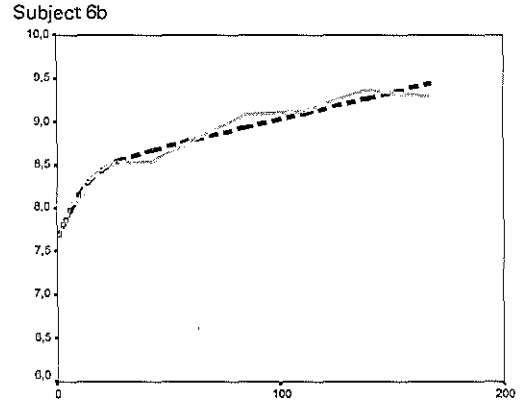

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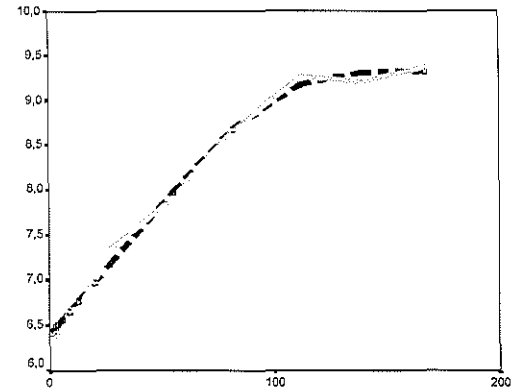

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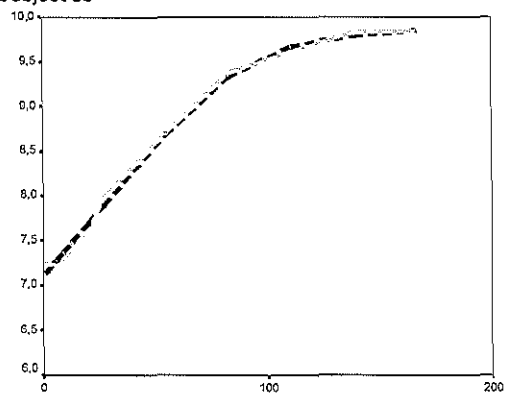

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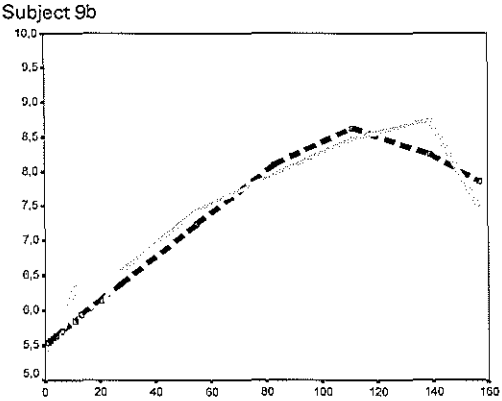

Discussion

The main action of nucleoside analogues is inhibition of viral replication through termination of the proviral chain. Lamivudine, which has been evaluated most extensively, has not shown to have any effect on cccDNA in *in vitro* systems (9,10). As a result, lamivudine therapy should be continued for a long time in order to be able to eliminate the virus through cell division and death of infected cells. It has been calculated that therapy should be continued for many years to achieve complete eradication of the virus from the liver (22). Unfortunately, indefinite prolongation of nucleoside analogue therapy will not be the answer to complete eradication of the virus due to a cumulative incidence of viral resistance (40-60% after two to three years of lamivudine monotherapy) (23-25).

As a result, one should aim for a compound which does exhibit two features: interference with viral replication as well as a reduction of infected hepatocytes. Entecavir has proved to cause minimal side-effects during short-term application (26) and *in vitro* data imply the possible effect on cccDNA (14). Therefore, therapy with this drug may reduce the amount of infected cells to a greater extent and in a shorter amount of time.

Although the results of this study are based on a small number of patients, the antiviral activity during short-term therapy with entecavir seems somewhat greater than during

lamivudine therapy (27) and lower than during adefovir dipivoxil therapy (17), although it should be realized that this is a head-to-head comparison and randomized studies are needed to identify the actual differences in parameters between these nucleoside analogues. Our analysis is based on four low doses of entecavir during the first study in chronic hepatitis B patients; some of these doses might have been insufficient for the optimal treatment of HBV. Moreover, the majority of our patients had previously failed lamivudine therapy which could also result in a less favorable response to re-introduction of another antiviral agent. Entecavir did show continuing activity in patients with detectable lamivudine-induced mutant virus. Theoretically, the second phase of more gradual decline in viral concentration may be influenced by death of infected hepatocytes and turn-over of cccDNA harboring cells. After the first 28 days of therapy, decline of viral concentration can be either slower than, equal to, or faster than observed during the second phase. We do not observe a difference in the slope of the second phase between lamivudine- and entecavir-treated patients in a head-to-head comparison. We could therefore speculate that in both lamivudine and entecavir treated patients, this second phase is primarily influenced by death of infected hepatocytes. If entecavir exhibits a direct effect on cccDNA, this effect may surface only during a longer treatment period.

All 9 patients who were evaluated after withdrawal of therapy showed a bi-phasic pattern with an initial fast increase of viral replication followed by a more gradual increase (subject 4,5 and 6) or a more or less steady state. This initial fast return of viral replication, which was calculated to last 30 days, could reflect the production capacity of the reservoir of hepatocytes that is still infected with HBV followed by the infection of non-infected hepatocytes leading to a larger productivity. This second episode is more pronounced in some patients than in others. The part of the cccDNA pool which was not affected by entecavir can be used as a template from which the virus can re-initiate replication once the inhibitor has been removed. This implies that a larger pool of still infected hepatocytes could result in a faster return of viral replication to baseline level. The second phase of this model represents a more or less steady state of viral production, counteracted by turn-over of free virus and infected hepatocytes.

Patients who were treated with the two higher doses of entecavir (0.5 and 1.0 mg) showed a more gradual increase in HBV DNA to baseline levels than those patients who were treated with lower doses, even though viral load was suppressed to the same extent in all dose groups. Slower return of viral replication in the high dosed groups may therefore be due to a smaller remnant pool of infected hepatocytes and not to the extent of viral suppression in serum. The latter explanation has previously been used as an explanation for the slower return of viral replication after withdrawal of lamivudine therapy (28).

In conclusion, these data show that both decay of viral concentration as well as rebound of hepatitis B viral concentration can be fitted with a mathematical model. Entecavir is effective in both patients infected with wildtype and variant hepatitis B virus. In the future, data on the actual amount of cccDNA in the liver of these entecavir treated patients could be helpful in supporting the outcome of the parameter estimates.

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9

DISCUSSION

A minority of chronic hepatitis B patients benefit from a four month course of alpha interferon (IFN-α) through induction of HBeAg seroconversion (1-3). Moreover, IFN-α therapy is also hampered by side-effects which require dose reduction in up to 20% of patients (4,5). Lamivudine, a recently registered, orally administered drug, shows a strong antiviral effect. Suppression of viral replication is pronounced in the majority of patients, resulting in normalization of serum transaminases and improvement of the histology activity index (HAI) on the liverbiopsy (6-8). However, monotherapy does have some definite disadvantages.
In this thesis we tried to identify and describe the drawbacks of lamivudine monotherapy and to explore the first steps of new treatment strategies able to overcome these disadvantages.

Modeling of viral dynamics
Comparison between individual patient data in response to antiviral therapy is most often based on the absolute decline in viral load (9) or the percentage of HBeAg seroconversion during a predetermined period of observation. (6). This type of evaluation, however, does not take into account the dynamic process that occurs during antiviral therapy. Multiple studies in hepatitis C virus (HCV) infected patients, based on mathematical modeling, have shown to be predictive of both viral clearance and the likelihood of sustained response (10,11). We, and others ((chapter 2,3,4,6 and 8 of this thesis), 12,13) have applied these pre-existing models to evaluate data in hepatitis B virus (HBV) infected patients during antiviral therapy. Surprisingly, the bi-phasic model which is based on data in HCV patients during IFN-α therapy (14) also describes the HBV data very well. Thus, the rough frame work of this model, which describes viral decline during a particular antiviral therapy is largely identical in both HBV and HCV infection. The hepatitis C virus, however, is a RNA-virus with a different replication cycle compared to HBV. Moreover, IFN-α inhibits viral replication and induces hepatotoxicity as well, unlike most nucleoside analogues evaluated in this thesis. Their primary function is inhibition of viral replication. This stresses the fact that the mathematical model describes viral decline accurately, regardless of type of infection or therapy used. It should therefore be emphasized that we can obtain parameters from the model which we can fit into the biological processes, but we should not try to fit the biological process into the model. It is necessary to keep on expanding the model with additional parameters when new data, based on basic biological research, become available. A recent study does indeed include non-cytolitic loss of hepatocytes (loss of cccDNA) into the model (15), but the value of this extra parameter cannot be calculated due to limitations in the technical options of the computerized models. Future molecular biologic studies could lead to even further expansion of the models. However, it is questionable whether this will provide us with a better understanding of the viral dynamic processes. We are both limited with regard to the technical applicability of the model and these extra parameters may not even change the values of the parameters which we can calculate at present (e.g. effectiveness in blocking viral production, turn-over of free virus and death rate of infected hepatocytes). So, at present the discussion focuses on leaving the model as it is, with all the limitations of this mathematical approach, or expanding the model making it more complex and more difficult to work with. Long-term follow-up data are needed, as in HCV modeling, to be able to relate initial response to therapy in the present model to long-term response in individual patients.

Patient monitoring
Some of the prerequisites for optimal modeling, in which we are different from the studies presented previously, should also be mentioned here. At first frequent well standardized quantitative HBV DNA determinations, are important for accurate modeling and should

become standard in all dynamic studies presented from this day onward; provided that this frequent sampling is achievable in clinical patient management. This will be the only way to be able to compare different studies with regard to the outcome of parameters. We would recommend minimal blood withdrawal at day 0, day 2, day 4 and day 7. In addition, all data in all patients should be analyzed simultaneously as the best statistical approach for comparison of groups of patients. All data presented so far (12,13,15), apply individual patient data from which mean group parameters are calculated. Since the outcome of both the group-wise analysis and the calculated mean of individual patients is similar (chapter 2), we suggest to use the approach of group-wise fitting in future studies.
As has been observed in previous lamivudine studies (16), baseline ALT has proven to be an important determinant of HBeAg seroconversion. We were the first to describe a relation between baseline ALT and death of infected hepatocytes by including this baseline parameter into the model. When these different approaches regarding response to antiviral therapy are combined, death of infected hepatocytes appears to be an important determinant of viral eradication. From these data, the primary goal of lamivudine seems to be to suppress the viral load adequately enough for this event to take place. Two studies in chronic hepatitis B patients however (17-18), state that pre-treatment ALT levels are inversely correlated to the emergence of drug resistant mutants. These conflicting data stress the importance of evaluation of larger groups of patients with regard to viral dynamic parameters. It also indicates the need for evaluation of similarities between viral dynamic studies and studies in which response to therapy is based on, for example, decline in HBV DNA and HBeAg seroconversion. These latter studies describe the relation between a baseline factor and an event that occurs during long-term therapy only. In analogy to studies in HCV infections, pre-therapy patient- and virus-characteristics become increasingly important. Recently, several studies on lamivudine therapy focussed on the response to lamivudine therapy and the emergence of mutations and their relation with viral subtypes or pre-existence of variant virus before start of therapy (19-21). Eventually this may lead to a tailor-made therapy approach in individual patients.

HBV combination therapies
Estimates of viral turn-over and the pre-treatment existence of variant virus in Human Immunodeficiency Virus (HIV) patients have shown that at least three different nucleoside analogues should be applied in order to be able to prevent the emergence of drug resistant mutants during antiviral therapy (22). In this thesis, we combined lamivudine with either famciclovir or ganciclovir in a small number of patients. These combinations, however, did not result in a more pronounced decline in viral replication than lamivudine alone. *In vitro* and *in vivo* studies on the combination of lamivudine and famciclovir (23-25) did show the superiority of lamivudine-famciclovir combination therapy over lamivudine monotherapy. This *in vivo* observed difference in response should however be interpreted with caution as the group of patients studied was small. Moreover, the majority of patients in our study had been pre-treated with lamivudine which may have caused a less favorable response to the addition of famciclovir because similar mutations in the polymerase of the HBV for lamivudine and famciclovir have been described (26). Therefore, it is of importance to combine nucleoside analogues of distinctive origin (cytosine, thymidine, guanine, adenine) in order to prevent competitive inhibition of the polymerase. In addition, analogues that are combined should not be capable of inducing the same mutations in the polymerase of the HBV. At this moment, this profile is met by entecavir and adefovir dipivoxil, which are both evaluated in clinical studies. Combinations of lamivudine and adefovir dipivoxil have been tested in *in vitro* systems and have proved to be synergistic (23). Adefovir dipivoxil expresses continuing activity against HBV variants induced by lamivudine (27-28). A first *in vivo* study

has also shown continuing effect of adefovir dipivoxil on lamivudine resistant HBV in liver transplant patients (29). However, present study designs aim at rescue therapy in patients who have already developed a lamivudine induced HBV variant. These patients will still be treated with one optimally active antiviral compound only. Therefore, future therapies should start with combinations of nucleoside analogues simultaneously and not in an overlapping "add-on" strategy. Combinations of lamivudine and equally or stronger virus suppressive agents like entecavir and adefovir dipivoxil are better options than famciclovir or ganciclovir, since the latter have only proved to be moderate suppressors of HBV replication if applied as monotherapy (30,31). The best option in the near future is either a combination of lamivudine and adefovir dipivoxil or adefovir dipivoxil monotherapy. Monotherapy with the latter nucleoside analogue may be an option since a recent study showed that after 1 year of therapy, the incidence of clinical relevant mutations is very low (32).

New nucleoside analogues that are under pre-clinical evaluation (L-FMAU) (33,34) or have just recently been applied in clinical trials (emtricitabine, FTC) could also be a candidate for combination therapy. L-FMAU is an uracil nucleoside analogue which was investigated for its effect on cccDNA in chronically infected woodchucks. Its EC_{50} proves to be a little less than lamivudine, but 10 times better than famciclovir. No effect on cccDNA other than the division and loss of infected hepatocytes during antiviral inhibition can be observed. FTC, a cytosine nucleoside analogue similar to lamivudine is also a strong virus suppressive agent (35,36). During 24 weeks of therapy, 25-100 mg of FTC induces 2.3-3.0 log reduction in viral replication with a favorable safety profile (37).

Complete eradication of the hepatitis B virus in once chronically infected patients can most probably not be achieved. Several studies in liver-transplantation patients and in patients who have cleared the virus from serum, have shown the persistent presence of low levels of HBV in the liver.(38-42). Induction of immunotolerance should therefore be the major goal of antiviral therapy in chronic hepatitis B patients. If this can be achieved, progression of the liverdisease will be prevented. Suppression of the virus to a large extend and for a long time may be followed by the natural turnover of infected hepatocytes which may eventually establish viral eradication. On the other hand, a suppressor of viral replication combined with an agent that has immunological capacity, could speed up the process of viral eradication.
Previous studies on lamivudine combined with alpha interferon in selected patients suggest the superiority of the combination therapy over either monotherapy alone (43,44). Present studies focus on the effect of the combination of lamivudine and PEG-interferon. PEG-interferon has the advantages of less frequent administration and supposedly fewer side-effect. This long-term combination therapy may enhance the HBeAg seroconversion ratio.
Entecavir, which is, like lamivudine, an inhibitor of viral replication, may have some direct effect on cccDNA as well (45,46). If this assumption can be proved during *in vivo* application, entecavir could directly eradicate the most stable form of the virus from the liver. Adefovir dipivoxil is known to induce stimulation of NK-cells which in turn could also result in a higher turnover of infected hepatocytes (47).

HIV-HBV co-infection
In this thesis, we describe a specific group of HIV-HBV co-infected patients, in which the emergence of mutations becomes increasingly important. A considerable percentage of immunocompetent patients develops a flare of serum transaminases (48). Although only one patient in our cohort experienced a hepatic flare without liver decompensation, recent reports (49,50) emphasize the fact that these flares may have serious consequences, even in this immunodeficient patient population. We determined a decrease in CD4-cells during HAART

as a factor correlated to the faster clinical detection of drug resistant mutants. A non-response to HAART should therefore be observed cautiously, and a switch to an alternative therapy (e.g. adefovir dipivoxil (51)) may be needed early during therapy. Whether one should start with lamivudine in this patient group, should probably be based on the same considerations as in immunocompetent patient. We observed more or less equal percentages of mutations in our patient group and HBV mono-infected patients. Therefore, the fear of occurrence of mutations should not primarily lead the decision whether to start with lamivudine or not. Lamivudine should be introduced in patients with active liver disease, in which the progression to cirrhosis could be prevented. On the other hand, one should probably not initiate lamivudine-therapy in co-infected patients with active liver disease in whom therapy for the HIV infection is not yet needed. Lamivudine monotherapy results in resistance of the HIV for lamivudine after a few weeks of therapy (52). This could hamper future HIV treatment strategies in these patients and this situation should, if possible, be avoided.

Finally

In conclusion, future treatment strategies in chronic HBV patients could include combination therapies with several nucleoside analogues, monotherapy with nucleoside analogues which have a direct effect on cccDNA in vivo or nucleoside analogue(s) in combination with an immunomodulating agent.

Frequent patient monitoring during the initial days of therapy is essential to arrive at a tailor-made therapy approach. Mathematical modeling, as described in this thesis, taking into account patient related factors like ALT and BMI can be used to select dosages of individual drugs, and to compare several combination therapies.

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10

SUMMARY
SAMENVATTING
DANKWOORD
CURRICULUM VITAE

Summary

Lamivudine, a suppressor of hepatitis B viral replication, has been studied intensively in several large studies. Recently, it has been licensed for its antiviral effect in patients with chronic hepatitis B infection. This antiviral effect may cause reduction of liver inflammation and result in an arrest of liver disease progression or even improvement of liver inflammation and fibrosis. In this thesis, we focussed on the possible causes of diminished response to lamivudine, on methods to evaluate the difference in response to nucleoside analogue therapy and we explored a number of ways to improve existing antiviral therapies.

Following the concept of oral applicable antiviral therapy, the search for new nucleoside analogues has been intensified and has resulted in many strong antiviral compounds. In the first chapter we describe the similarities and differences between five nucleoside analogues which are applied in clinical studies. These nucleoside analogues are capable of interfering with the replication of the hepatitis B virus because of their resemblance to natural nucleosides. However, this quality also enables them to induce serious adverse events. Therefore, it is important to focus on new antiviral compounds which exhibit a positive balance between effectiveness and safety.

A mathematical model has been applied to describe hepatitis C viral decline during interferon therapy. In chapter 2 we use this same bi-phasic model to describe hepatitis B viral decline during lamivudine therapy and compare this model with an exponential model and a bi-phasic model with a flat second phase. The latter two models proved to be less accurate. For evaluation, a group-wise analysis is the best statistical approach due to population interactions. Differences in results between individual fitting and group-wise fitting were minimal. In chapter 3, high and low dose of lamivudine were compared with the bi-phasic model. The baseline factors ALT and previous lamivudine therapy were significantly related to viral decline during the second phase and viral decline during the first phase respectively.

Since the reasons behind a suboptimal response to lamivudine are not clear, we studied the first step in the pharmacokinetic process of lamivudine therapy. Lamivudine levels in blood of patients who participated in a previous study on the efficacy of lamivudine, were compared with a reference curve. Concentrations of lamivudine 6 hours after intake in the study group were within the range of the reference curve. Further research into the concentrations of active lamivudine-triphosphate inside the hepatocyte is needed to elucidate the causes of differences of viral decline between patients (chapter 4).

In chapter five we describe the emergence of mutations which is one of the known causes of a diminished response to lamivudine. Since the Human Immunodeficiency Virus (HIV) infected population shows a prolonged survival after the introduction of Highly Active AntiRetroviral Therapy (HAART), more focus should be placed on the Hepatitis B Virus (HBV) as an important factor of co-morbidity. We report on two HIV-HBV co-infected patients; one patient developed a mutation to lamivudine during prolonged lamivudine therapy and the other a hepatitis flare after the withdrawal of lamivudine. These cases show the importance of monitoring both HBV and HIV infection carefully (chapter 5A). In a multi-center study, a cohort of HIV-HBV co-infected patients on long-term lamivudine therapy was evaluated for the emergence of mutations. The calculated incidence of 25% at one year and 52% at two years is roughly similar to the immunocompetent patient population. Baseline Body Mass Index and decline of CD4 cells during therapy were significantly related to the emergence of mutations (chapter 5B). In the last part of this chapter we describe the virus suppressive potential of famciclovir in one patient who had developed a YIDD mutation during long-term lamivudine therapy (chapter 5C).

Because of the emergence of mutations during long-term lamivudine therapy and the probable failure of lamivudine to eradicate the HBV from the liver, there is a need to explore new

treatment strategies. First of all, we applied combinations of nucleoside analogues to aim at more pronounced virus suppression or to prevent the emergence of mutations. We evaluated the response to lamivudine monotherapy, lamivudine-ganciclovir and lamivudine-famciclovir combination therapy by means of the bi-phasic model. No significant difference between the three groups was observed. This may be explained by the sample size or the duration of therapy but our impression is that these combinations are not of major importance compared to lamivudine monotherapy (chapter 6).

Thereafter, we studied a new nucleoside analogue, entecavir, with potential effect on covalently closed circular DNA (cccDNA), the most resistant form of the HBV inside the liver. We evaluated entecavir in a one month, placebo-controlled, dose-escalating study on the safety and efficacy of four doses of entecavir. All doses (0.05 mg, 0.1 mg, 0.5 mg and 1.0 mg) resulted in a pronounced decline of HBV DNA (2.21, 2.25, 2.81 and 2.42 log respectively) (chapter 7).

Patients in the previous study who were treated in our hospital also participated in an ancillary study on viral dynamics. We evaluated the dynamics of viral decay of the HBV during 28 days of entecavir and the return of viral replication until 6 months after withdrawal of entecavir therapy. Both viral decay and viral rebound are bi-phasic; calculation of dynamic parameters could support the biological phenomena that occur during and after withdrawal of antiviral therapy (chapter 8).

At the moment, combinations of antiviral drugs are being evaluated for the treatment of chronic HBV infections, similar to the development of combination therapies for the HIV infection a few years ago. Mathematical modeling taking into account differences in baseline parameters like ALT and previous treatment is capable of describing the difference between treatment strategies, as well as clarifying the mechanisms which are responsible for the reaction to the different antiviral drugs. Modeling could be a useful tool to eventually discover the most appropriate therapy for chronic hepatitis B patients.

Samenvatting

Lamivudine is een geneesmiddel dat de vermenigvuldiging van het hepatitis B virus (HBV) remt. Het middel is uitgebreid onderzocht in verscheidene grote studies. In 2000 is het geregistreerd als behandeling van chronische hepatitis B patiënten. Door de virusonderdrukkende werking kan de leverontsteking verbeteren, de progressie van de leverziekte worden voorkomen of zelfs de mate van fibrose afnemen. In dit proefschrift hebben wij ons gericht op de mogelijke oorzaken van een verminderde respons op lamivudine, op methodes om het verschil in respons op therapie met nucleoside analoga te evalueren en op het verder verbeteren van de reactie op bestaande en nieuwe antivirale therapie.
Oraal toe te dienen medicatie heeft voordelen boven parenterale toediening en in dit licht is er intensief gezocht naar therapie in tabletvorm, wat heeft geresulteerd in een groot aantal krachtige antivirale middelen. In het eerste hoofdstuk beschrijven we de overeenkomsten en verschillen tussen de vijf belangrijkste orale nucleoside analoga die worden gebruikt in klinische studies. Deze nucleoside analoga zijn in staat om de vermenigvuldiging van het HBV te remmen omdat ze grote gelijkenis vertonen met natuurlijke nucleosiden. Deze eigenschap zorgt echter ook voor ernstige bijwerkingen. Daarom is het belangrijk om uit te kijken naar nieuwe antivirale middelen met een positieve balans tussen effectiviteit en veiligheid.
Een wiskundig model is gebruikt om virus-afname tijdens interferon-behandeling voor hepatitis C patiënten te beschrijven. In hoofdstuk 2 maken we gebruik van dit twee-fase model om de afname van het hepatitis B virus tijdens lamivudine-behandeling te beschrijven en om het model te vergelijken met een exponentieel model en een twee-fase model met een horizontale tweede fase. De laatste twee modellen leverden de minst precieze beschrijving op. Om virusafname van groepen patiënten te beschrijven, is groepsgewijze analyse de beste statistische methode voor evaluatie van patiëntenpopulaties in verband met populatie-interacties. Het verschil tussen individuele "fits" en groepsgewijze "fits" blijken minimaal. In hoofdstuk 3 zijn een hoge en een lage dosis lamivudine vergeleken met behulp van het twee-fase model. De uitgangswaarden ALT en eerdere lamivudine-behandeling waren significant gerelateerd aan virusafname tijdens respectievelijk de tweede en eerste fase.
Omdat de oorzaken van sub-optimale respons op lamivudine behandeling niet geheel duidelijk zijn, hebben we de eerste stap in het pharmacokinetische proces van lamivudine bestudeerd. Lamivudine spiegels in bloed van patiënten die eerder deelnamen aan een studie over de effectiviteit van lamivudine, werden vergeleken met een referentiecurve. Concentraties van lamivudine zes uur na inname in de studiegroep en de referentiegroep lagen binnen dezelfde grenzen. Verder onderzoek naar concentraties van de actieve metaboliet lamivudine-trifosfaat, is nodig om de oorzaken van sub-optimale respons op lamivudine behandeling in bepaalde patiënten op te helderen (hoofdstuk 4).
In hoofdstuk vijf beschrijven we het optreden van mutaties, een van de bekende redenen van een verminderde respons op lamivudine. Omdat de overleving van de Human Immunodeficiency Virus (HIV) geïnfecteerde populatie na de introductie van Highly Active AntiRetroviral Therapy (HAART) langer is geworden, dient meer aandacht te worden besteed aan een hepatitis B infectie als een belangrijke factor van co-morbiditeit. We beschrijven twee HIV-HBV gecoïnfecteerde patiënten: een patiënt ontwikkelde een HBV-mutatie tegen lamivudine na langdurige lamivudine-behandeling en de ander een hepatitis flare na staken van lamivudine behandeling. Deze casus benadrukken het belang van goede controle van zowel de HIV als de HBV infectie (hoofdstuk 5A). In een multi-center cohort studie hebben we een cohort van HIV-HBV gecoïnfecteerde patiënten die gedurende langere tijd met lamivudine werden behandeld, geëvalueerd voor het optreden van mutaties. De berekende

incidentie van 25% na 1 jaar en 52% na twee jaar zijn grofweg hetzelfde als in de niet-immuun gecompromitteerde patiënten populatie. Baseline Body Mass Index en afname van CD4 cellen tijdens behandeling waren beiden significant gerelateerd aan het optreden van mutaties (hoofdstuk 5B). In het laatste deel van dit hoofdstuk beschrijven we de virusonderdrukkende potentie van famciclovir in een patiënt die een YIDD mutatie had ontwikkeld tijdens langdurige lamivudine behandeling (hoofdstuk 5C).
Vanwege het optreden van mutaties tijdens langdurige lamivudine-behandeling en het mogelijke onvermogen van lamivudine om het HBV ccc-DNA te verwijderen uit de lever, is het noodzakelijk om nieuwe behandelingsstrategieën te ontwikkelen. In eerste instantie hebben we combinaties van nucleoside analoga toegepast om een krachtiger onderdrukking van het virus te bewerkstelligen en het ontstaan van mutaties te voorkomen. We evalueerden de respons op lamivudine-monotherapie, lamivudine-ganciclovir- en lamivudine-famciclovir-combinatie-therapie met behulp van het twee-fase model. Er werd geen significant verschil tussen de verschillende groepen gevonden. Dit zou kunnen worden verklaard door de beperkte groepsgrootte of de korte duur van behandeling maar onze indruk, gebaseerd op deze beperkte dataset, is dat deze combinaties niet van groot klinisch belang zijn in vergelijking met lamivudine monotherapie (hoofdstuk 6).
Daarna bestudeerden we een nieuw nucleoside analogon, entecavir, welke mogelijk effect heeft op de meest resistente vorm van het HBV in de lever: het covalently closed circular DNA (cccDNA). We onderzochten entecavir in een 1 maand durende, placebo-gecontroleerde, dosis-oplopende studie naar de effectiviteit en veiligheid van vier doseringen van entecavir. Alle doseringen (0.05 mg, 0.1 mg, 0.5 mg en 1.0 mg) resulteerden in een uitgesproken onderdrukking van het HBV DNA (2.21, 2.25, 2.81 en 2.42 log respectievelijk) (hoofdstuk 7).
Patiënten in de voorafgaande studie die in ons ziekenhuis werden behandeld namen ook deel aan een studie over virale dynamiek. We evalueerden de virusafname gedurende 28 dagen behandeling met entecavir en de terugkeer van virusvermeerdering tot 6 maanden na staken van entecavir-behandeling. Zowel virusafname als terugkeer van virusvermeerdering vertonen elk een twee-fasen verloop. Berekening van dynamische parameters zou de biologische verschijnselen die optreden tijdens en na behandeling kunnen ondersteunen (hoofdstuk 8).
Conform de ontwikkeling van antivirale therapie voor HIV infecties, worden op dit moment de eerste combinaties van virusonderdrukkende middelen voor HBV-infecties geëvalueerd. Wiskundige modellering, rekening houdend met populatieverschillen in serum ALT en eerdere behandeling objectiveert de verschillen tussen de behandelingen en verheldert mogelijk de biologische mechanismen die ten grondslag liggen aan de respons op de verschillende antivirale middelen. Op deze manier gebruikt kan de modelmatige benadering een bruikbaar hulpmiddel zijn om uiteindelijk tot een optimale behandelingsstrategie voor chronische hepatitis B patiënten te komen.

Dankwoord

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Curriculum Vitae

De auteur van dit proefschrift werd geboren op 27 juli 1970 in Maassluis. In 1988 behaalde zij het Gymnasium B eindexamen aan het Christelijk Lyceum Gouda waarna zij gedurende een jaar heeft gestudeerd aan Lawrence University, Appleton, Wisconsin, USA. In 1989 is zij begonnen met de opleiding Geneeskunde aan de Erasmus Universiteit te Rotterdam. Aan het einde van de doctoraal fase heeft zij haar afstudeeronderzoek verricht in het pharmacologische laboratorium van de Erasmus Universiteit (Prof. Dr. P.R. Saxena). Het eerste deel bestond uit proefdieronderzoek waarbij gekeken werd naar het effect van bacteriën op het ontstaan van inflammatoire darmziekten; het tweede deel betrof onderzoek bij gezonde proefpersonen naar het effect van nicotine op de productie van verscheidene cytokines die betrokken zijn bij inflammatoire darmziekten. Voordat zij in 1995 begon met de co-schappen heeft zij gedurende een maand stage gelopen in het Christian Medical College, Vellore, India. Vervolgens werd in 1997 nog een keuze-coschap gevolgd op de afdeling Leverziekten en Levertransplantatie in het Royal Prince Alfred Hospital in Sydney, Australië. Sinds 1997 heeft zij, onder begeleiding van Dr. R.A. de Man en Prof. Dr. S.W. Schalm, onderzoek verricht naar de behandeling van chronische hepatitis B patiënten op de afdeling Maag-, Darm- en Leverziekten (hoofd Prof. Dr. E.J. Kuipers) van het Dijkzigt Ziekenhuis, Rotterdam. Dit onderzoek vormt de basis van dit proefschrift. In januari 2001 is zij gestart met de vooropleiding interne geneeskunde in het Bronovo Ziekenhuis, Den Haag (opleider Dr. J.W. van 't Wout), waarna zij in mei 2004 de opleiding tot Maag-, Darm- en Leverarts zal vervolgen in het Dijkzigt Ziekenhuis, Rotterdam (opleider Prof. Dr. E.J. Kuipers).

Abbreviations

3TC	2'3'-dideoxy-thiacytidine (lamivudine)
AIC	Akaike's Information Criteria
AIDS	Acquired ImmunoDeficieny Syndrome
ALT	alanine aminotransferase
AST	aspartate aminotransferase
AUC	area under the curve
AZT	zidovudine
bis-POM-PMEA	bis(pivaloyloxymethyl)-(9-(2-phosphonylmethoxyethyl)adenine)
BMI	body mass index
CC_{50}	50% cytotoxic concentration
cccDNA	covalently closed circular DNA
CI	confidence interval
CI_{50}	50% inhibitory concentration
CMV	cytomegalovirus
ddC	zalcitabine
dGTP	deoxyguanine 5'-triphosphate
DR1	direct repeat region 1
DR2	direct repeat region 2
dTTP	deoxythymine 5'-triphosphate
EC_{50}	50% effective concentration
geq	genome equivalents
γGT	gamma glutamyl transferase
HAART	Highly Active AntiRetroviral Therapy
HAV	hepatitis A virus
HBV	hepatitis B virus
HBeAg	hepatitis B envelop antigen
HBsAg	hepatitis B surface antigen
HCV	hepatitis C virus
HDV	hepatitis D virus
HIV	human immunodeficiency virus
HPLC	high pressure liquid chromatography
IU	international units
Ki	inhibitory constant
Km	Michaelis constant
Ki/Km	ratio which expresses competitive inhibition of a nucleoside analogue and a natural nucleoside
MEq	mega equivalents
mRNA	messenger ribonucleic acid
NNRTI	Non-Nucleoside Reverse Transcriptase Inhibitor
od	once daily
PBMC	peripheral blood mononuclear cells
PCR	Polymerase Chain Reaction
PEU	Paul Ehrlich Institute Units
PT	prothrombin time
s.d.	standard deviation
s.e.	standard error
T½	half-life
ULN	Upper Limit of Normal
UV	ultra violet
WHV	woodchuck hepatitis virus
YIDD	thyrosine isoleucine aspartate aspartate
YMDD	thyrosine methionine aspartate aspartate
YVDD	thyrosine valine aspartate aspartate