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Short communication

Correlation between entecavir penetration in peripheral blood mononuclear cells and HBV DNA decay during treatment of HBeAg-negative chronic hepatitis B

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ABSTRACT

Background: Recently, due to its high effectiveness and tolerability, the treatment of chronic hepatitis B with entecavir became a standard practice. However, limited knowledge is currently available about its pharmacokinetic behavior and intracellular disposition. Recently, our group reported an inverse correlation between entecavir plasma concentrations and the HBV DNA decay at the first and third month of treatment, respectively.

In this paper we investigated the disposition of entecavir in peripheral blood mononuclear cells (PBMC) and in plasma, in order to evaluate the relationship between intracellular penetration and response, in a cohort of naïve patients with HBeAg-negative CHB.

Methods: Thirty-tree patients were prospectively enrolled and gave written informed consent: the monitoring of clinical parameters (e.g. HBV DNA, HBsAg, ALT, etc.) was carried out at the baseline and then monthly. Entecavir intra-PBMC and plasma trough concentrations were measured at 1 month of treatment, through a validated method based on liquid chromatography coupled with tandem mass spectrometry.

Results: While plasma entacavir analysis confirmed previous evidence of inverse correlation between drug concentrations and HBV DNA decrease after 3 months of treatment ($r=-0.723$; $p<0.001$), this correlation was not significant for intra-PBMC concentrations. When the intracellular disposition ratio (intra-PBMC/plasma concentration ratio) was considered, it showed a direct and significant correlation with HBV DNA decay at the third month ($r=0.485$; $p=0.004$).

Conclusions: These results suggest that the antiviral activity of entacavir is dependent on its intracellular uptake, thus resulting in lower plasma concentrations in patients who have a marked HBV DNA decrease during treatment.

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Running head: Entecavir intracellular penetration and antiviral efficacy.

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INTRODUCTION

Chronic hepatitis B (CHB) is among the major causes of liver cirrhosis, liver failure and hepatocellular carcinoma, affecting about 257 million people worldwide [1].

Therapeutic options include long-term therapy with oral nucleos(t)ide analogues (NAs) and the finite-duration therapy with pegylated interferon alfa (PEG-IFNα) [2]. However, treatment with PEG-IFNα has limited efficacy and is poorly tolerated, whereas NAs-based treatments are limited by the indefinite therapy duration and by the onset of resistance [3].

Currently, the most used NAs are entecavir (ETV) and tenofovir disoproxil fumarate (TDF), however, these treatments are rarely associated with loss of HBsAg, especially in HBeAg-negative patients [2]. In fact, the monitoring of quantitative serum HBsAg (qHBsAg) during treatment with NAs evidenced a slower decrease compared to IFN therapy [4,5], while the long-term HBV-DNA suppression is obtained in almost all patients treated with ETV or TDF [3].

Up to now, information about the concentration-effect relationships for ETV has been scarce; this is probably due to the high effectiveness of ETV in the longer term, making the study of early viral kinetics less attractive. However, considering new insights on protocols for a finite treatment duration on entecavir, including sequential or combined therapy with PEG-INFα [6–9], this topic is becoming more important.

Recently, an inverse correlation between ETV plasma trough concentrations and the decrease of HBV DNA after 3 months of treatment was reported [10]. This peculiar phenomenon was hypothesized to be due to the effect of hepatic uptake (especially the first-pass) on ETV plasma concentrations: higher hepatic uptake would result in stronger antiviral effect and lower plasma exposure. Differences in hepatic uptake can underlie variability in liver perfusion, genetic differences in drug transporters or the presence of co-medications.

In support of this model, other drugs (e.g. pravastatin) acting on liver show a similar behavior [11–13]: nevertheless, this was never observed before for antivirals.

Since direct evaluation of ETV disposition in hepatocytes requires liver biopsy, too invasive to be performed without indication, in this work we evaluated ETV penetration in peripheral blood mononuclear cells (PBMC). In fact, although hepatocytes and PBMC are obviously different in terms of expression of drug transporters and in their physiology, some transporters already known to be involved in ETV disposition in liver [14,15] are also expressed in lymphocytes [16,17].

METHODS

Patients and clinical monitoring

Naïve HBeAg-negative patients with active CHB treated with ETV in our centre at the University Hospital “Amedeo di Savoia” (Turin, Italy), were prospectively enrolled in this approved pharmacokinetic study (“HBV ANALOGUES”, n° 4/2015, 1/26/2015, “Amedeo di Savoia” hospital Ethics Committee), giving written

informed consent. Naïve HBeAg-negative patients, without decompensated cirrhosis, HCC or co-infections were included.

Before treatment, the following tests were performed: evaluation of liver stiffness by Fibroscan®, HBV genotype testing, liver ultrasonography, ALT, qHBsAg and HBV DNA quantification.

ETV was administered at the standard dose of 0.5 mg once-daily.

After treatment beginning, HBV-DNA, qHBsAg and ALT levels were monitored monthly for the first three months, then every six months. On the other hand, ETV quantification in plasma was performed at the first and third month of treatment, while ETV quantification in PBMC was performed only at the first month.

Virological and Serological Assays

Serum HBV-DNA was quantified by Real Time PCR COBAS AmpliPrep/COBAS TaqMan HBV Test 2.0 (Roche Molecular Systems, NJ, USA). HBV genotypes were determined by INNOLIPA reverse hybridization assay (Innogenetics, Belgium). HBsAg, HBeAg and anti-HBe were detected by the Elecsys instrumental platform (Roche Diagnostics, Italy); qHBsAg test was performed with ARCHITECT HBsAg (Abbott Diagnostics, Ireland).

Blood sampling, plasma and PBMC isolation and ETV quantification

Blood samples were collected 24h after the last drug intake, immediately before the next administration (C_{trough}). In order to obtain plasma, blood was collected in lithium heparin tubes (7ml) and centrifuged 1400 *x g* for 10 min at +4 °C: then plasma samples were stored at -20 °C until the analysis (less than 1 month). ETV quantification in plasma was performed following a previously published UHPLC-MS/MS method [18].

In addition, PBMC were isolated from blood using CPT vacutainers [19]. Briefly, blood samples were centrifuged at 1700 *x g* for 15 minutes, then the PBMC layer was transferred to a Falcon tube and washed twice with isotonic saline. During this process PBMC were counted and measured with an automated cell counter, in order to determine the exact volume of cells. Each sample was divided in two aliquots: one was treated with acid phosphatase [19,20], in order to convert the phosphorylated intracellular metabolites of ETV (mono-, di- and tri-phosphate) in free ETV, the other one was left untreated. Both aliquots were stored at -80°C until analysis.

ETV quantification in PBMC was performed following a previously published UHPLC-MS/MS method coupled with on-line solid phase extraction [20].

Statistical analysis

Data were assessed for normality with Shapiro-Wilk test. Binomial groups were compared exploiting Mann-Whitney test; Pearson correlation tests were performed to identify correlations between continuous variables. Finally, a ROC curve analysis was conducted to find concentration cut-off values. Independent predictivity of factors associated with the HBV DNA decay kinetics was evaluated through univariate and multivariate linear regression analysis.

All statistical analyses were performed using SPSS program version 22.0.

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RESULTS

Baseline patients features

Thirty-three CHB-patients were included in this analysis. Baseline characteristics of patients are reported in Table 1. Patients were ethnically mixed and infected with different HBV genotypes.

ETV plasma and intra-PBMC concentrations

Median ETV plasma concentration after 1 month of standard treatment was 0.346 ng/mL (IQR 0.252 – 0.623), while the median concentration of total ETV (phosphorylated and unphosphorylated forms) in PBMCs was 83.8 ng/mL (IQR 61.7 – 109.9). Free ETV was always undetectable in PBMCs. Median intra-PBMC/plasma concentration ratio was 241.3 (IQR 141.7 – 298.8) at one month of treatment. After 3 months, median ETV plasma concentration was 0.467 ng/mL (IQR 0.310 – 0.692).

ETV concentration-response relationship

Significant inverse correlations between ETV plasma concentrations at 1 month and HBV DNA Log decay after 1 month ($r=-0.581$; $p<0.001$), 2 months ($r=-0.694$; $p<0.001$) and 3 months ($r=-0.723$; $p<0.001$) of treatment have been observed. Conversely, intra-PBMC ETV concentrations at 1 month of treatment did not significantly correlate with HBV DNA during treatment (p between 0.1 and 0.3). However, the intra-PBMC/plasma ETV concentration ratio did correlate with HBV DNA Log decay after 1 month ($r=0.375$; $p=0.032$), 2 months ($r=0.401$; $p=0.021$) and 3 months of treatment ($r=0.485$; $p=0.004$; Figure 1). No significant correlation has been observed between ETV levels in plasma and PBMC with HBsAg Log decay during treatment ($r=-0.217$; $p=0.178$).

Prediction of viral DNA decay kinetics

By univariate regression, ETV plasma concentration ($p<0.001$) and the intra-PBMC/plasma concentration ratio ($p=0.004$) after 1 month of treatment were found to be significant predictors of HBV DNA Log decay after 3 months. After multivariate analysis, ETV plasma concentration resulted the strongest predictor ($p<0.001$).

ROC curve analysis indicated ETV intra-PBMC/plasma concentration ratio higher than 250 as a significant predictor of a HBV DNA decay higher than 2 Logs ($p<0.001$; sensitivity 60.0%; specificity 84.6%): positive predictive value (PPV) resulted 85.7% and negative predictive value (NPV) was 57.8% (Figure 1). All patients reached virological response later on treatment.

DISCUSSION

In this work, both ETV plasma concentration and the intra-PBMC/plasma concentration ratio showed significant correlation with the HBV DNA Log reduction during treatment in an inverse and direct way, respectively.

By contrast, the intra-PBMC total ETV concentration showed no significant correlation with either HBV DNA Log decay and ETV plasma concentration, confirming that the inter-individual differences in ETV penetration in PBMC would be based on mechanisms of active transport and accumulation.

The evidence of a direct and strongly significant correlation between the accumulation ratio and HBV DNA Log levels suggests that more efficient mechanisms of active transport of ETV within cells from plasma could underlie a stronger antiviral affect.

However, although PBMCs and hepatocites share the expression of transporters already known to affect ETV uptake and efflux, such as OCT1/2 and MDR family [15–17], this happens in the systemic circulation, explaining the lack of correlation of intra-PBMC ETV concentrations with HBV DNA decay: in fact, although this relationship is not linear, by a theoretical point of view a low systemic ETV plasma concentration, expected to be related to higher penetration in liver, also reduces the total concentration in PBMC.

According to this observation, ETV plasma concentrations are better predictors of HBV DNA decay after 3 months than intra-PBMC/plasma concentration ratio.

Considering these evidences, intra-PBMC ETV quantification appears to be only a confirmative and elucidating tool in cases of very low ETV plasma concentration. In fact, in these cases the interpretation of data would be ambiguous: low plasma concentrations could underlie high ETV intracellular uptake, and so high response, or low therapeutic adherence, and so low response.

Considering this issue, intracellular quantification would be useful to help distinguish between these two possibilities. Nevertheless, since no cases of poor adherence have been observed in this study (naïve patients with strong motivation for treatment), further studies are needed to confirm this possible use of intracellular ETV quantification.

Conflicts of interest

No sources of potential conflict of interest to be declared.

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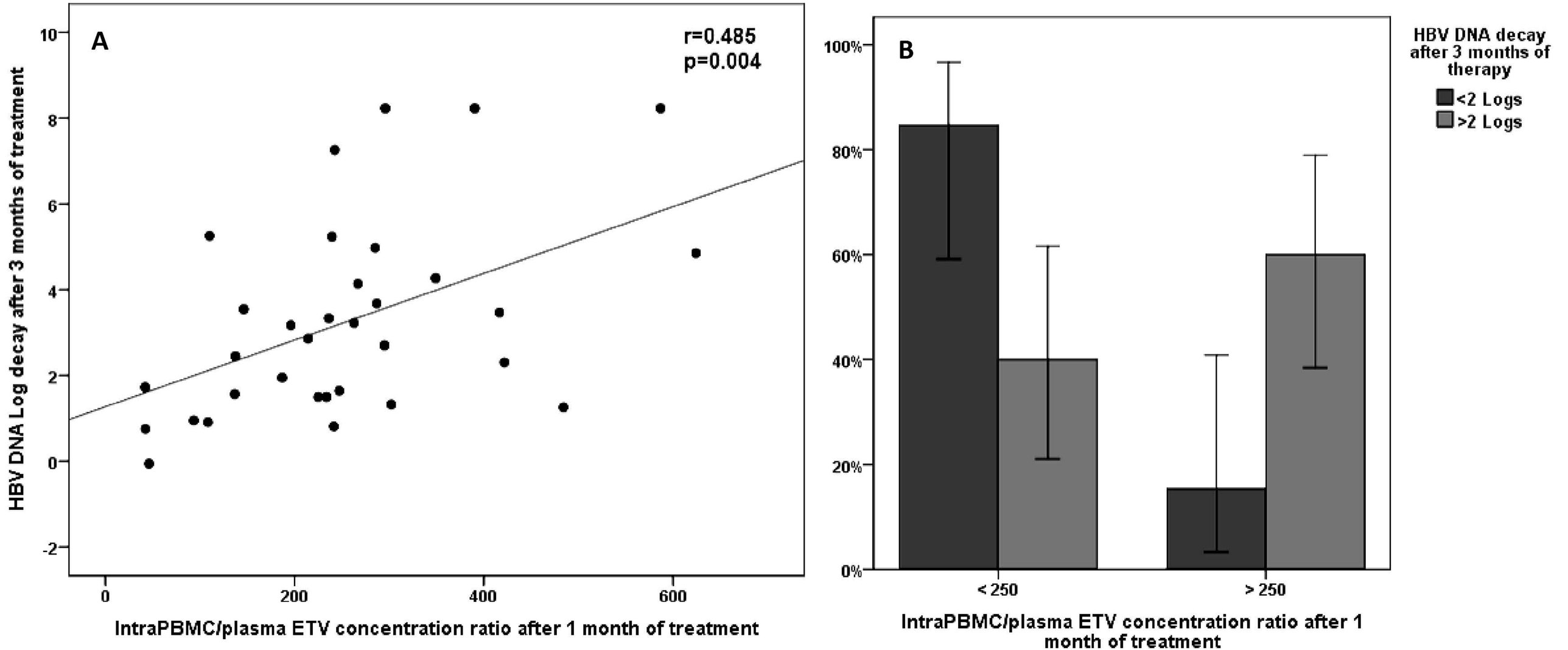
Figure Legends

Figure 1: (A) Direct correlation between intraPBMC/plasma concentration ratio after 1 month and HBV DNA decay after 3 months of treatment. (B) Percent distribution of cases of a virological response after 3 months of treatment in patients with an intraPBMC/plasma ETC concentration ratio higher or lower than the cut-off value of 250. Error bars indicate the 95% confidence interval.

Table 1. Baseline features of included patients.

Patients features	**n=33**
Age (yr) median[IQR]	42 [28 - 53]
Male sex n (%)	27 (81.8)
Liver stiffness (kPa) median [IQR]	6.1 [5.6 – 9.8]
qHBsAg (log IU/mL) median [IQR]	4.01 [3.83 - 4.8]
HBV-DNA (log IU/mL) median [IQR]	6.94 [5.91 - 8.55]
ALT (U/L) median [IQR]	93 [74.2 - 134]
HBV genotypes n (%)	A: 16 (48.5) B: 3 (9.1) C: 3 (9.1) D: 9 (27.3) E: 2 (6.0)


A
r=0.485
p=0.004
HBV DNA Log decay after 3 months of treatment
10
8
6
4
2
0
-2
0
200
400
600
IntraPBMC/plasma ETV concentration ratio after 1 month of treatment
B
100%
80%
60%
40%
20%
0%
< 250
> 250
IntraPBMC/plasma ETV concentration ratio after 1 month of treatment
HBV DNA decay after 3 months of therapy
<2 Logs
>2 Logs