**Phyllanthus amarus** suppresses hepatitis B virus by interrupting interactions between HBV enhancer I and cellular transcription factors

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Abstract. The **Phyllanthus amarus** plant suppresses HBV mRNA transcription in vitro and exhibits therapeutic potential in chronic HBV carriers, although further work is necessary to define its mechanism of action. Analysis in HuH-7 cells with transfected plasmids using a luciferase reporter showed that *P. amarus* specifically inhibited HBV enhancer I activity. To identify the mechanism of this HBV enhancer I inhibition, liver-enriched cellular transcription factors were co-expressed in HuH-7 cells. The C/EBPα and β, as well as HNF-3α and β transcription factors, significantly up-regulated the HBV enhancer I activity. In contrast, co-transfection of HNF-1α or β had no effect upon the HBV enhancer I activity. Exposure to *P. amarus* inhibited C/EBPα- and β-mediated up-regulation of HBV enhancer I activity in a dose-dependent manner, whereas HNF-3α- and β-mediated up-regulation of HBV enhancer I was unaffected. In vitro gel shifts showed that *P. amarus* inhibited complexing of C/EBP transcription factors to a consensus oligonucleotide sequence, whereas DNA binding of AP-1 and SP-1 transcription factors was unaffected. As *P. amarus* down-regulates HBV mRNA transcription by a specific mechanism involving interactions between HBV enhancer I and C/EBP transcription factors, purification and further analysis of the active *P. amarus* component will advance insights into its antiviral activity.

Keywords. Hepatitis B virus, liver, medicinal plant, treatment.

Introduction

Hepatitis B virus (HBV) afflicts approximately 300 million people worldwide, with the spectrum of liver disease encompassing hepatitis, cirrhosis and hepatocellular carcinoma. Despite significant mechanistic insights into HBV replication and gene expression, satisfactory therapies are lacking [1]. Several years ago, crude extracts of the **Phyllanthus amarus** plant were found to inhibit HBV polymerase activity and to clear HBsAg in chronic HBV carriers [2,3]. Our own studies using the HepG2 2.2.15 cells, which support HBV replication, and G26 HBV transgenic mice, which express HBsAg independently of other viral proteins or HBV replication, showed that *P. amarus* extracts decreased virion production and down-regulated HBsAg mRNA transcription, including suppression of the viral glucocorticoid-responsive element [4]. These findings raised important questions concerning whether *P. amarus* regulated the HBV enhancers and whether this was a direct vs. an indirect effect.

The HBV enhancer I (HBEnI) is the better characterized of the two known HBV enhancers and regulates all four open reading frames of the virus [5,6]. The HBEnI contains binding sites for multiple transcription factors, including AP-1, nuclear factor-1 (NF-1), hepatocyte nuclear factor (HNF)-3, CCAAT/enhancer-binding proteins (C/EBPs) and members of the steroid/thyroid nuclear receptor superfamily (e.g. HNF-4), but not for others, such as HNF-1 [7–10]. The presence of multiple transcription factor-binding domains in DNA sequences is most consistent with their regulatory roles as subunits, although the precise contribution of individual transcription factors is often difficult to determine because unique inhibitors are lacking. The hepatotropism of HBV is directed by cell surface receptors regulating viral entry and also by cellular transcription factors governing viral gene expression, because HBV expression is limited to specific epithelial cells in liver and other organs, such as the kidneys, pancreas and stomach [11,12]. As cellular transcription factors are developmentally regulated in specialized epithelia, the lineage relationship between cells and specific transcription factors probably determines the permissivity for HBV gene expression in tissues [13,14].

We hypothesized that the mechanism as to how *P. amarus* regulated the HBEnI could be determined in cell culture using plasmid transfection assays. As a paradigm, we cloned an expression plasmid containing the HBEnI and the pre-S1 HBV promoter. Additional promoters regulating other HBV genes, such as core and X genes,
are also recognized, although the pre-S1 promoter exhibits significant tissue type specificity [15]. The goals of our studies were to define whether the down-regulatory effect of *P. amarus* was specific to the HBEI, to examine whether *P. amarus* interfered with HBEI and cellular transcription factor interactions and to demonstrate whether *P. amarus* exerted its activity by either interrupting transcription factor binding to HBEI or decreasing the availability of specific transcription factors.

**Materials and methods**

*P. amarus*

Plants harvested in Tamilnadu, India, by S.P.T., were solubilized in dimethyl sulfoxide (DMSO) as reported previously [4]. Briefly, the plant (4 g) was crushed with a blender in 45 mL of deionized water and stirred overnight at room temperature. Undissolved material was pelleted in glass tubes at 1000 × g for 20 min at 25 °C and solubilized in 5 mL of DMSO (Sigma Chemical Co., St Louis, MO, USA). The supernatant and DMSO-solubilized material were pooled, diluted fourfold in normal saline, passed through a 0.45-μm filter and stored at 4 °C until use. The final extract was estimated to contain 5% DMSO and approximately 20 mg mL⁻¹ *P. amarus*.

**Cells**

The HuH-7 cells were derived from a human hepatocellular carcinoma and belong to the hepatocyte lineage [16]. Cells were cultured in RPMI-1640 medium containing 10% fetal bovine serum (FBS), 100 U mL⁻¹ penicillin and 100 μg mL⁻¹ streptomycin. For analysis, cells were exposed to 50, 100 or 200 μg mL⁻¹ *P. amarus* or to equivalent amounts of the vehicle, DMSO.

**Expression plasmids**

*HBV luciferase plasmids*. The plasmid, pCP10, which contains a head-to-tail HBV dimer [17], provided the sequence containing the HBV enhancer I (HBEI, 912–915 bp) with the pre-S1 promoter (HBpreS1P, 514-bp Bgl II fragment spanning baseregars 490–1402) and the pre-S1 promoter (HBpreS1P, 514-bp Bgl II fragment spanning baseregars 2425–2839) for cloning into pGL2-luc vectors (Promega, Madison, WI, USA). The plasmids designated pGL2.HBpreS1P/HBEI-luc and pGL2.SV40P/HBEI-luc contained the HBEI upstream to HBpreS1P or the simian virus 40 promoter (SV40P). The plasmid pGL2.HBpreS1P/SV40En-luc contained the SV40 enhancer (SV40En) upstream of HBpreS1P.

*Control luciferase plasmids*. The plasmid pGL2.control containing SV40 promoter and enhancer was a positive control, and the plasmid pGL2.basic with no regulatory elements was a negative control.

*Other reporter plasmids*. The plasmid pALb-CAT contains a chloramphenicol acetyltransferase gene driven by the mouse albumin promoter and enhancer [18]. The plasmid pSVβgal contains the SV40 promoter and enhancer driving the *Escherichia coli lacZ* gene (Promega).

**Transcription factors**. Plasmids expressing HNF1α and β, HNF3α and β and C/EBPα and β were kindly provided by either Dr G. Crabtree, Stanford University or Dr R. Costa, University of Illinois [19,20]. The HNF-5α and β and C/EBP α and β plasmids were regulated by the CMV promoter and HNF1α and β by the SRα promoter.

**Cell transfections**

One day after plating 5 × 10⁴ HuH-7 cells cm⁻² in medium containing FBS, cells were washed twice with phosphate-buffered saline (PBS), pH 7.4, before adding 1 μg of plasmid cDNA (≈ 90% supercoiled) and 5 μL of Lipofectin (Gibco BRL, Gaithersburg, MD, USA), which were preincubated in serum-free Optimem medium (Gibco BRL). Transcription factor plasmids and reporter plasmids were co-transfected in 1:5 or 1:10 ratios. After incubating cells with plasmids for 6 h, fresh RPMI-1640 medium containing *P. amarus* was switched and cells cultured for an additional 48 h before analysis. At least five separate experiments were performed for each condition.

**Reporter assays**

*Luciferase activity*. Cells were lysed and processed using a commercial kit (Promega). Cellular protein in the lysates was measured using the Bio-Rad assay (Bio-Rad, Hercules, CA, USA), and the results expressed as arbitrary light units mg⁻¹ protein.

*CAT activity*. Transfected cells were lysed by three freeze–thaw cycles in 0.25 mol L⁻¹ Tris-hydrochloride, pH 7.8. After pelleting cell debris, CAT activity was assayed in the supernatant as described previously [21].

**LacZ activity**. Cell monolayers were fixed with 0.5% glutaraldehyde in PBS and incubated for 6 h with X-gal (5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside) solution at 37 °C [22]. Cells staining blue were counted in 10 randomly selected high-power fields and expressed as a percentage of the total cell count. LacZ activity was enzymatically assayed by incubating cell lysates in 120 mmol L⁻¹ NaHPO₄, 80 mmol L⁻¹ Na₂HPO₄, 2 mmol L⁻¹ MgCl₂ and 100 mmol L⁻¹ β-mercaptoethanol with 1.33 mg mL⁻¹ o-nitrophenyl β-d-galactopyranoside (Sigma) followed by spectrophotometry at 420 nm [23]. The data were normalized to total protein content in cell lysates.

**Cell viability**

The cell number and exclusion of 0.2% Trypan blue dye were manually determined using a haemocytometer. Use of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium
bromide dye (MTT) was analysed after exposing cells to 1 mg mL$^{-1}$ MTT in phenol red-free minimal essential medium (MEM) for 90 min at 37 °C (Gibco BRL). The blue formazan product was solubilized in propanol and optical density measured at 560/690 nm [24].

Nuclear protein isolation and gel mobility shift assay

The probes were: C/EBP oligonucleotides, consensus, 5'-TGCAGATTGCCGAATCTGCA; mutant, 5'-TGCAAGACTAGTCTCTGCA (Santa Cruz Biotechnology, Santa Cruz, CA, USA); AP-1 consensus oligonucleotide, 5'-CGCTTGATGAGTCAGCCGGAA; and SP-1 consensus oligonucleotide, 5'-ATTCACTGGGCGGGCCGAGC (Promega). The oligonucleotides were labelled with $\gamma^32P$-ATP using polynucleotide kinase to specific activities of 10$^7$ cpm g$^{-1}$ (Boehringer Mannheim, Indianapolis, IN, USA). Nuclear extracts were prepared from isolated rat hepatocytes by the method of Dignam et al. [25], and the concentration of nuclear proteins was measured using the Bio-Rad assay with bovine serum albumin standards. Gel shifts with AP-1 and SP-1 oligonucleotides used HeLa cell nuclear extracts (Promega). Radiolabelled oligonucleotides were incubated with 6 µg of nuclear extract each in the presence of 1 µg of poly[dI–dC] for 20 min at room temperature. DNA–protein complexes were resolved in 4% polyacrylamide gels in 0.5·Tris borate–EDTA buffer.

Statistical analysis

Data are expressed as means ± SEM with significance determined by Student's $t$-tests. $P < 0.05$ was considered significant.

Results

P. amarus is non-toxic to cells

To demonstrate whether changes in gene expression or regulation could simply be accounted for by cell toxicity,

![Figure 1](image1.jpg)  
**Figure 1.** Effect of *P. amarus* upon cell viability. When HuH-7 cells were incubated with various concentrations (up to 500 µg mL$^{-1}$) of *P. amarus* for 72 h as shown here, or longer, there was no change in either the cell number or MTT utilization by cells, indicating an absence of cytotoxicity.

![Figure 2](image2.jpg)  
**Figure 2.** Gene expression in transient transfection assays. (A) Analysis of the relative transcriptional efficacy of the HBEnl and pre-S1 promoter and SV40 enhancer and promoter in HuH-7 cells showed that luciferase activity was most efficiently expressed by the homologous SV40 regulators (pGL2.control plasmid), followed by the heterologous unit containing the HBEnl and SV40 promoter, which was several-fold more potent than the pGL2.HBpreS1P/HBEnl sequence. The SV40 enhancer and pre-S1 promoter combination was the least efficient and pGL2.basic devoid of any promoters showed no luciferase expression. (B) In the presence of *P. amarus*, luciferase expression driven by the pre-S1 promoter/HBEnl combination was most affected, with suppression by up to 50%. *P. amarus* also suppressed luciferase expression driven by the SV40 promoter and HBEnl combination, although the magnitude of suppression was less. In contrast, the pre-S1 promoter/ SV40 enhancer containing plasmid or the pGL2.control plasmid were unaffected by *P. amarus*.

HepG2 2.2.15 cells were incubated for 72 h with medium containing 100, 200 or 500 µg mL$^{-1}$ *P. amarus* (Fig. 1). The cell viability was also unchanged in response to *P. amarus* treatment in additional experiments conducted after exposing cells to up to 500 µg mL$^{-1}$ *P. amarus* for 5 days, along with unchanged cell morphology (not shown). In addition, *P. amarus* did not change lacZ expression after transfection of HuH-7 cells with pSVβgal, which resulted in similar numbers of X-gal positive blue cells in control and test conditions (2.4 ± 0.4% vs. 2.2 ± 0.5%, $P = NS$). The lacZ activity measured by enzymatic assays was also similar in cells exposed to 500 µg mL$^{-1}$ *P. amarus* (control, 0.13 ± 0.01 lacZ units mg$^{-1}$ protein h$^{-1}$; test, 0.10 ± 0.01 lacZ units mg$^{-1}$ protein h$^{-1}$, $P = NS$). Use of more than one parameter for cell viability provided rigorous evidence of no
change in cell membrane integrity, metabolic capacity and transcriptional activity after exposure to *P. amarus*. Our previous studies showed that *P. amarus* did not interfere with cell proliferation mechanisms during log-phase cell growth [4].

**P. amarus shows specificity for HBEnI compared with SV40 enhancer in HuH-7 cells**

The SV40 enhancer and promoter most efficiently expressed luciferase, followed by the HBEnI and SV40 promoter combination, which was several-fold more potent than the HBEnI and pre-S1 promoter combination, as well as the SV40 enhancer/pre-S1 promoter, although the activities of the last two combinations were also readily apparent (Fig. 2A). The findings indicated that homologous regulatory sequences were more effective than heterologous ones and that the SV40 sequences were more potent than the HBV sequences in HuH-7 cells. Vehicle alone containing equivalent concentrations of DMSO, by itself, had no effect upon the expression of reporter plasmids (data not shown).

*P. amarus* dose dependently inhibited HBEnI and pre-S1 promoter by up to approximately 50% of the controls, *P* < 0.001 (Fig. 2B). In addition, *P. amarus* significantly suppressed the HBEnI/SV40 promoter combination (*P* < 0.03), although the magnitude of suppression was less, most possibly because of differences in the regulation of pre-S1 and SV40 promoters. In contrast, luciferase plasmids driven by the SV40 enhancer were unaffected by *P. amarus*. These results demonstrated that the down-regulatory effect of *P. amarus* was specific to the HBEnI, as the SV40 sequences or the HBV pre-S1 promoter were spared. However, whether *P. amarus* affected the HBV sequences alone, altered the binding of cellular transcription factors with HBV sequences or decreased the availability of cellular transcription factors required further studies.

![Figure 3](image-url)  
**Figure 3.** Regulation of HBEnI activity by cellular transcription factors. Luciferase activity 48 h after transfection of the pGL2.HBpreS1P/HBEnI-luc in HuH-7 cells was 1.1 ± 0.3 × 10^6 light units mg^-1 protein (100%). The pGL2.HBpreS1P/HBEnI-luc plasmid was maximally up-regulated by HNF-3α (323 ± 51% of control), followed by C/EBPα (314 ± 33%), C/EBPβ (260 ± 0%) and HNF-3β (158 ± 14%), whereas neither HNF-1α nor β showed any up-regulation.

![Figure 4](image-url)  
**Figure 4.** Regulation of the mouse albumin promoter and enhancer in HuH-7 cells. The cells were transfected with the palb.CAT plasmid, and CAT activity was assayed as described in Materials and methods after culture for 48 h. Lane 1, chloramphenicol alone; lane 2, co-transfection with HNF-3β at 1:5 molar ratio; lane 3, co-transfection with HNF-3β at 1:10 molar ratio; lane 4, palb.CAT alone; lane 5, co-transfection with HNF-3β at 1:10 molar ratio; and Lane 6, co-transfection with C/EBPα at molar ratio of 1:10. Note that gene expression was up-regulated by co-transfection with HNF-1 and C/EBP plasmids.

*Interactions between HBV enhancer, cellular transcription factors and *P. amarus***

To test the hypothesis that *P. amarus* inhibited HBEnI by modulating transcription factor interactions, co-transfection experiments with pGL2.HBpreS1P/HBEnI-luc and HNF-3α or β or C/EBPα or β were performed in HuH-7 cells. The ability of these transcription factor plasmids, particularly HNF3α and C/EBPs, which are liver enriched, to up-regulate the HBV enhancer I activity was clearly shown (Fig. 3). HNF-1α and β had no significant effect upon luciferase activity, although a specific binding site is thought to exist in the pre-S1 promoter sequence cloned in our pGL2.HBpreS1P/HBEnI-luc plasmid [26]. To eliminate the possibility that our HNF-1 plasmid was not functionally intact in HuH-7 cells, we analysed up-regulation of the mouse serum albumin promoter and enhancer, which contain HNF-1-binding sites [27]. When the palb-CAT plasmid was co-transfected with transcription factor plasmids, the mouse serum albumin enhancer and promoter were up-regulated by HNF-1, as well as HNF-3α and C/EBPα (Fig. 4).

The addition of *P. amarus* to HuH-7 cells transfected with pGL2.HBpreS1P/HBEnI-luc plasmid effectively suppressed the HBEnI activity to only 20 ± 4% of controls with a dose of 200 µg mL^-1^ (Fig. 5). Moreover, *P. amarus* also markedly inhibited the up-regulation of HBEnI activity by co-expressed C/EBPα or β transcription factors. In fact, the effect of *P. amarus* upon C/EBPα-mediated HBEnI up-regulation was selective because *P. amarus* decreased HBEnI activity only marginally when the pGL2.HBpreS1P/HBEnI-luc plasmid was co-transfected with either HNF3α or β. These findings suggested that C/EBPs play a predominant role in regulating the HBEnI, which would also be consistent with the epithelial cell-type restriction of HBV expression, as shown previously [12]. We expected no change in HuH-7 cells

exposed to *P. amarus* after co-transfection with pGL2.HBpreS1P/HBEnI-luc and HNF-1 plasmids, as the latter were ineffective in up-regulating HBEnI. Although these experiments clearly demonstrated that *P. amarus* could interfere with interactions between HBV and cellular transcription factors, it was unclear whether the mechanism involved the occupation of DNA-binding sites by *P. amarus* components or the decreased availability of transcription factors, e.g., by degradation or inactivation, transcriptional down-regulation or altered post-transcriptional processing. One way to investigate these possibilities was to perform gel shift assays to show whether, in the presence of preformed transcription factors, *P. amarus* interfered with transcription factor binding to specific consensus DNA sequences.

P. *amarus* inhibited binding of C/EBPs to a consensus sequence

Gel-shift assays used nuclear extracts from hepatocytes, which contain abundant C/EBPs, or HeLa cells, which offer an excellent system for analysing general transcription factor activity, including for AP-1 and SP-1. The assays were performed in the presence of either *P. amarus* or the vehicle alone. The studies showed that C/EBPs in hepatic extracts could no longer bind and shift their consensus DNA sequence in the presence of *P. amarus* (Fig. 6A). This effect of *P. amarus* was dose dependent. In contrast, there was no C/EBP binding to a mutant oligonucleotide, which served as a negative control.

Finally, assays were performed to determine whether
the inhibitory effect of *P. amarus* was specific for C/EBPs. When binding of AP-1 or SP-1 to their consensus DNA sequences was tested with HeLa cells extracts, gel shifts were readily observed (Fig. 6B). AP-1 is an ubiquitous transcription factor and represents a heterodimer of the early activated cell cycle-regulated c-fos/c-jun gene product. SP-1 is also extensively distributed, with binding domains present on a wide variety of cellular and viral promoter elements, although its regulatory role may be dependent upon the presence or absence of additional transcription factor subunits. However, *P. amarus* showed no effect upon the binding of either AP-1 or SP-1, which was further consistent with a specific effect of *P. amarus* upon C/EBP binding.

Discussion

The data demonstrate in several ways that *P. amarus* down-regulates HBV by specifically inhibiting HBEnI. The initial evidence of such an inhibitory activity in *P. amarus* was generated by our experiments using HepG2 2.2.15 cells, which support HBV replication [28]. In these cells, exposure to *P. amarus* resulted in decreased steady-state HBV mRNA levels and abolition of dexamethasone-mediated up-regulation in HBV mRNA transcription, indicating an inhibitory effect upon the glucocorticoid-responsive element of the viral enhancer [4,29]. Secondly, our data here show that *P. amarus* inhibited HBEnI activity in transient plasmid-based expression systems, but did not affect the SV40 enhancer in independent reporter systems. Finally, we found that *P. amarus* interfered with the regulatory axis between HBEnI and cellular transcription factors by inhibiting DNA binding. Despite our use of crude *P. amarus* in enormous concentrations, there was no evidence of cell toxicity, as shown by Trypan blue dye exclusion, cell number changes or MTT utilization.

The relative specificity of the inhibitory effect of *P. amarus* on HBEnI activity may be caused, at least partly, by cell type-specific mechanisms regulating the HBEnI enhancer in liver cells [12]. Although the SV40 enhancer, as well as some other viral regulators, are broadly active in a variety of cell types, the HBEnI is extinguished in cells of non-epithelial origin and even in permissive epithelial cells, such as hepatocytes, renal tubular cells, gastric epithelial cells, pancreatic acinar cells, etc., significant differences are apparent in the magnitude of gene expression. In transient cell transfection assays, the HBEnI was appropriately regulated by HNF-3α and C/EBPα transcription factors in hepatocytes but not in non-parenchymal epithelial liver cells or fibroblasts, which indicates that transcription factors subserve critical roles in regulating the HBEnI activity. Our data suggest that, among various transcription factors capable of regulating HBV, C/EBPs are dominant. The C/EBPs, which are enriched in hepatocytes and participate in terminal differentiation programmes [14], serve as important transcriptional modulators [30]. Proteins of the C/EBP family accumulate preferentially in non-mitotic, terminally differentiated liver cells and regulate multiple liver-specific genes, including the serum albumin gene. This might well be why HBV is expressed in cells of epithelial origin in various organs that are presumably permissive for C/EBP activity [12].

Mapping of the pre-S1 promoter has shown binding sites for HNF-1, as well as HNF-3, which enhance its activity in hepatocytes [31,32], although we were unable to show an up-regulatory effect of HNF-1 upon our pGL2.HBpreS1P/HBEnI-luc plasmid. Our findings here are in agreement with non-deleterious down-regulation by *P. amarus* of serum albumin mRNA levels in HepG2 2.2.15 cells [4], which also probably resulted from interference with cellular transcription factor-mediated up-regulation, because the serum albumin gene is regulated by HNF-1, C/EBPs and other transcription factors [27]. Interestingly, *P. amarus* did not influence transcriptional regulation of the HBEnI by HNF-3α and β transcription factors, which indicated selectivity of the drug. However, the HNF-3s were less potent than C/EBPs in regulating the HBEnI because *P. amarus* did not affect HNF-3-mediated up-regulation of the viral enhancer but suppressed enhancer activity by virtually up to 70% of the controls, presumably through its inhibitory effects upon C/EBPs. It was helpful to show that interference by *P. amarus* of C/EBP binding to DNA was a specific effect because *P. amarus* did not interfere with the binding of SP-1 and AP-1 transcription factors. The HBEnI does contain an AP-1-binding site, but our data suggest that AP-1 must not play a significant modulatory role in HBV expression because *P. amarus* did not affect its binding to DNA and yet markedly decreased HBEnI activity. Similarly, the SP-1 transcription factor binds to a variety of viral and cellular promoters, but *P. amarus* had no effect on altering its DNA-binding capacity. Such clear demonstrations of transcription factor interactions *in vivo* have not been possible previously because specific inhibitors were not available. Therefore, abrogation by *P. amarus* of C/EBP binding to DNA offers specially intriguing possibilities for further dissection of general transcriptional mechanisms, as well as the regulation of HBV.

We excluded inadequate intracellular expression of the transfected HBV-1 plasmid in our system, because the HNF-1α and β plasmids could up-regulate the mouse serum albumin promoter/enhancer in HuH-7 cells. The HBEnI contains additional transcription factor-binding domains, such as the one capable of binding a liver-specific factor and a ubiquitous transcription factor [32], which is believed to be EF-C or RFX1 belonging to a dimerizing family of nuclear proteins [33]. These too were less important in HuH-7 cells. The gel shift assays strongly suggested to us that *P. amarus* blocked interference with HBV DNA binding sites to C/EBPs. Such a mechanism will also help to reconcile why *P. amarus* has been found to suppress HBV DNA polymerase activity *in vitro* [2,4]. Our *in vitro* HBV polymerase assays were based upon the principle of primer extension using virion particles containing the entire genome [4]. If the primer DNA sequences were rendered unavailable after binding of *P. amarus* to HBV DNA, the assay would show
inhibition of DNA polymerase activity. This, rather than enzymatic mechanisms, most probably reconcile our findings concerning the inhibition of the HBEnI, as well as viral polymerase activity, because P. amarus remains active despite treatments, such as extensive heating, that eliminate the activity of most proteins, although further work will be necessary to prove this possibility. Of course, inhibition of the HBEnI may also down-regulate polymerase gene transcription and decrease viral replication in vivo.

In view of the ubiquitous regulatory roles of the HBEnI, inhibition by P. amarus of the enhancer should profoundly affect the viral life cycle. We believe that the findings have important translational connotations. In chronic HBV carriers, liver injury occurs during periods of viral replication or reactivations, during which specific viral proteins induce a host immune response [35,36]. If the viral load in treated patients were to diminish progressively or an imbalance in viral gene expression the viral load in treated patients were to diminish, viral proteins induce a host immune response [35,36]. If the viral load in treated patients were to diminish, viral proteins induce a host immune response.

Our in vitro assays shall greatly facilitate the isolation of the active P. amarus component that interferes with HBV transcription. Purification of the active ingredient is necessary for the systematic and accurate analysis of the antiviral potential of P. amarus.

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