the HBV infection experiments. These data suggest that CsA blocked HBV infection.

Effect of cyclosporin A on HBV entry

CsA decreased HBs and HBe secreted from the infected cells in a dose-dependent manner (Fig. 2A). We next investigated which step in the HBV life cycle was blocked by CsA. The HBV life cycle can be divided into two phases: the early phase of infection including attachment, entry, nuclear import, and cccDNA formation, and the following late phase representing HBV replication that includes transcription, assembly, reverse transcription, and viral release (32). Lamivudine drastically decreased HBV DNAs in HepAD38 cells (33), which reproduce HBV replication but not the early phase of infection (Fig. 2B). In addition, continuous treatment with lamivudine as well as entecavir and interferon-α for 4 days after HBV infection could decrease HBV DNA levels in HBV-infected HepaRG cells, which suggests an inhibition of HBV replication (Fig. 2C). Nevertheless, lamivudine did not show an anti-HBV effect when applied only prior to and during HBV infection (Fig. 1A and B), suggesting that anti-HBV compounds identified in Fig. 1A interrupted the early phase of the HBV life cycle.

We then examined whether CsA inhibited attachment or entry. For evaluating HBV attachment (34), cell surface HBV DNA was extracted and quantified from HepaRG cells exposed to HBV at 4°C for 3 h and then washed (Fig. 2D-a). For the internalization assay (34), the above cells, after washing, were further cultured at 37°C for 16 h to allow HBV to internalize into the cells, and then trypsinized to digest HBV remaining on the cell surface to allow quantification of internalized HBV DNA (Fig. 2D-b). CsA slightly reduced the amount of attached HBV DNA, although the effect was not statistically significant (Fig. 2D-a). In contrast, CsA caused a significant reduction of HBV DNA in the internalization assay (Fig. 2D-b). In a time of addition assay as shown in Fig. 2E, treatment with CsA during HBV infection decreased HBs and HBe production (Fig. 2E-b), while CsA did not have an anti-HBV effect when delivered after HBV infection (Fig. 2E-c). Thus, CsA appears to primarily block the entry step including internalization. To examine whether CsA targeted HBV particles or host cells, we preincubated HBV with CsA and then purified away CsA from the HBV inoculum, followed by the measurement of the HBV infectivity using HepaRG cells (Fig. 2F). Preincubation with CsA did not affect HBV infectivity in contrast to the

antagonizing effect of heparin to HBV particles (Fig. 2F), suggesting that CsA did not affect HBV particles but rather targeted host cells.

Cyclosporin A showed a pan-genotypic anti-HBV effect

We examined the anti-HBV effect of CsA on the infection of different genotypes of HBV into primary human hepatocytes. As shown in Fig. 3A, CsA reduced the infection of HBV genotype A, C, or D, which differ in sequences from the virus strain used in all of the other figures. However, CsA did not affect the entry of HCV, in contrast to the inhibition of HCV entry by heparin, bafilomycin A1, or an anti-HCV E2 antibody (Fig. 3B).

Effect of immunosuppressants on HBV infection

CsA is used clinically as an immunosuppressant, such as in patients following liver transplantation (13). We therefore investigated the activity of other immunosuppressants on HBV infection. Among the additional immunosuppressive drugs examined, only FK506 was able to suppress HBV infection (Fig. 4A). CsA is known to have three major cellular targets: cellular cyclophilins (CyPs), calcineurin (CN), and transporters including MDRs and MRPs (18). Although both CsA and FK506 can inhibit CN (Fig. 4B), this activity was dispensable for the anti-HBV effect, as PSC833, a CsA derivative inactive for CN inhibition (Fig. 4B) (18), could still inhibit HBV infection (Fig. 4C). As PSC833 and FK506 did not bind to the active site of CyPs (Fig. 4D), CyP inhibition is not likely to be responsible for the anti-HBV activity.

CsA blocked HBV infection through targeting NTCP

Recently, NTCP was reported as a candidate entry receptor for HBV (22). A transporter activity assay showed that CsA inhibited the activity of NTCP both in 293 (Fig. 5A) and HepG2 cells (Fig. 5B) engineered to stably overexpress NTCP, as previously reported (35). CsA was also suggested to bind to NTCP on the membrane in a ligand binding assay using HepG2-NTCP cells (Fig. S2).

NTCP mRNA was expressed in HepaRG cells and PHH, which are HBV susceptible, while little to no expression was detected in HBV non-susceptible cell lines including HepG2, Huh-7, FLC4, and non-hepatocyte HeLa cells (Fig. 5C). In contrast, CyPA and CyPB were

expressed in all of these cell lines, irrespective of infection susceptibility. Intriguingly, we found that the inhibition of NTCP transporter activity correlated with anti-HBV entry activity (Fig. 5A and Fig. 4A and B). These results suggest the possibility that compounds targeting NTCP have the potential to block HBV infection. To test this prediction, we treated HepaRG cells with compounds known to inhibit NTCP, including ursodeoxycholate, cholic acid, propranolol, progesterone, and bosentan (35, 36) to investigate the effect on HBV entry using the protocol in Fig. 1A. As shown in Fig. 5D, these compounds inhibited HBV infection. Thus, inhibition of NTCP blocked HBV infection. We also showed that HepG2 cells overexpressing NTCP were susceptible to HBV infection (Fig. 5E), as reported recently (22). Treatment with CsA also reduced HBs and HBe secretion when these cells were infected with HBV (Fig. 5E), suggesting that CsA inhibited NTCP-mediated HBV infection.

The binding of the HBV large envelope protein (LHBs) to NTCP was reported to be important for HBV entry (22). Thus one mechanism by which compounds that directly inhibit NTCP activity may block HBV entry is interruption of the binding between NTCP and LHBs. To test this possibility, we established an AlphaScreen assay to evaluate LHBs-NTCP binding in vitro as described in Experimental Procedures. In vitro synthesized NTCP and LHBs were at least partially functional, as NTCP bound to its substrate TCA (Fig. S3A) and LHBs could neutralize HBV infection into HepaRG cells (Fig. S3B). As shown in Fig. 5F, incubation of recombinant NTCP with LHBs but not middle (MHBs) and small envelope protein (SHBs) produced a significant AlphaScreen signal (Fig. 5F-a, left) indicative of direct protein-protein interaction. In contrast to NTCP, recombinant GST or other non-relevant proteins, LCK and FYN (37) did not produce a binding signal when incubated with LHBs (Fig. 5F-a), suggesting that our AlphaScreen assay produced a specific signal for the interaction of NTCP with LHBs. Consistent with the report that the pre-S1 region of LHBs was important for the binding to NTCP (22), the signal was decreased in a dose-dependent manner by the addition of pre-S1 lipopeptide HBVpreS/2-48^{myr} (5) (Fig. 5F-b) but not of an inactive mutant of pre-S1 (Fig. S3C), indicating a competition of pre-S1 with LHBs for NTCP binding. In this assay, CsA as well as FK506 and a CsA derivative, SCYX1454139 (see the next section), were shown to reduce the signal for LHBs-NTCP binding in a dose-dependent manner (Fig. 5F-c, d, and e). These results suggest that CsA targets NTCP and thereby inhibits the interaction between LHBs and NTCP.

Identification of cyclosporin A analogs possessing a higher anti-HBV potential

Considering CsA as a lead compound, we tested CsA analogs for anti-HBV activity. As shown in Fig. 6A, SCYX618806 reduced HBs secretion after HBV infection, while a related analog SCYX1774198 did not have a significant anti-HBV effect (Fig. 6A and C). Additional analogs, SCYX827830 and SCYX1454139, had significant anti-HBV activities (Fig. 6A and C). Alisporivir (Debio 025), an anti-HCV drug candidate (38), also decreased HBV infection to the equivalent level to CsA (Fig. 6B). Fig. 6D shows dose-dependent reduction of HBs secretion by treatment with SCYX618806, SCYX827830, and SCYX1454139, all of which had more potent anti-HBV activities than CsA (compare Fig. 6D with Fig. 2A). These results indicate that anti-HBV activity is not disrupted by at least some changes to the 3-glycine, 4-leucine, and 8-alanine residues of CsA, although additional analogs will need to be evaluated for a full understanding of structure-activity relashionships. Notably, SCYX618806 and alisporivir bear modifications on the 4-leucine residue of the CsA backbone that prevent CN binding and immunosuppressive activity (Table S1), further confirming that anti-HBV activity does not require immunosuppressive activity. Notably, SCYX1454139 showed the strongest anti-HBV entry activity among 50 CsA derivatives examined (data not shown and Fig. 6E). The IC_{50}s for anti-HBV activity as well as CC_{50}s determined by a MTT-based cell viability assay are shown in Fig. 6E. The IC_{50} and CC_{50} of SCYX1454139 were 0.17±0.02 and >10 μM, respectively, a profile superior to that of CsA (IC_{50} and CC_{50} of 1.17±0.22 and >10 μM, respectively). Inhibition of HBV infection by treatment with SCYX1454139 was also observed in primary human hepatocytes, in which also the anti-HBV effect of SCYX1454139 was more remarkable than that of CsA (Fig. 6F). These results clearly indicate that analogs of CsA may include compounds with greater anti-HBV potency than that of CsA itself.

Discussion

Previous reports have demonstrated that CsA suppresses the replication of a variety of viruses including human immunodeficiency virus, HCV, influenza virus, severe acute respiratory syndrome coronavirus, human papillomavirus, flaviviruses, vesicular stomatitis

virus, and vaccinia virus (16, 39-46). Virological analyses using CsA further demonstrate that CyPs are involved in the replication of these viruses. In this study, we showed that CsA inhibited the entry of HBV but in an apparent CyP-independent manner. It was previously reported that CsA suppressed HBV replication in a cell culture system carrying an HBV transgene (47). However, this anti-replication effect is not likely to be responsible for the anti-HBV activity observed in this study, based on several observations. Firstly, the experimental system mainly used in this study (Fig. 1A) is likely to evaluate the early phase of HBV infection but not HBV replication. Secondly, the suppression of HBV replication by CsA reported previously was mediated by blocking the mitochondrial permeability transition pore possibly through binding to mitochondrial CyPD (47, 48). The anti-HBV activity shown in this study, however, had no correlation with binding to CyPs, suggesting that the inhibition of HBV infection in HepaRG cells and primary human hepatocytes is not from the result of suppression of HBV replication. Rather, CsA inhibited NTCP transporter activity and disrupted the binding between NTCP and LHBs in vitro. Moreover, inhibition of HBV infection could be observed by treatment with other compounds having the capacity to inhibit NTCP. These results suggest that targeting NTCP blocks HBV infection.

The current anti-HBV agents are mainly comprised of nucleos(t)ide analogues and IFNs. Development of anti-HBV agents targeting different molecules is greatly needed for achieving improved treatment of HBV infection, especially to combat drug resistant virus. HBV cell entry mechanisms have been poorly defined. At the initial stage, HBV attaches to target cells with low affinity through binding involving cellular factors including heparan sulfate proteoglycans (28, 29). For the subsequent entry mechanism, it has recently been reported that NTCP is essential for HBV specific entry (22). Although the precise mechanism for entry and internalization is as yet incompletely understood, interference with this step has emerged as an attractive approach for development of novel therapeutics. For example, Gripon et al. demonstrated that a peptide mimicking the pre-S1 region of large envelope protein prevented HBV infection in a mouse model (5). These results suggest that inhibition of virus cell entry could be an effective strategy for preventing HBV infection to achieve clinical outcomes such as for post-exposure prophylaxis, blockage of vertical transmission, and prevention of HBV recurrence after liver transplantation. Given that HBV reactivation generally occurs under immunosuppressive conditions (49, 50), it is uncertain

whether clinically relevant doses of CsA or FK506 could be helpful in preventing HBV reactivation after liver transplantation. It remains also unknown in general whether entry inhibitors could be effective in eliminating chronic HBV infection. Future studies should evaluate whether inhibition of HBV entry by CsA or its derivatives can reduce persistent HBV infection, especially in combination with nucleos(t)ide analogues or interferons. In this study, we obtained non-immunosuppressive CsA derivatives that could inhibit HBV entry (Fig. 6). Moreover, a small scale analog analysis identified a CsA derivative exhibiting more potent inhibition of HBV infection, with as IC_{50} of 0.1-0.2 μM (Fig. 6). This IC_{50} is equivalent to the anti-HCV replication activities of alisporivir or SCY-635 (0.22 μM and 0.08 μM, respectively), drugs which have been shown to reduce HCV viral load in infected-patients during clinical trials (38). Further analog analysis using CsA as a platform may identify more potent anti-HBV compounds.

In general, antiviral drugs targeting a cellular factor select drug resistant virus at a lower frequency than do direct-acting antiviral agents. Cellular targets relevant for anti-HBV drug development have been poorly defined to date. This study has demonstrated that small molecules targeting NTCP can inhibit HBV infection. Further study of NTCP inhibitors and CsA derivatives may provide a new anti-HBV strategy targeting a cellular factor, which is less likely to foster emergence of drug resistant virus.

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Figure Legends

Fig. 1. Cyclosporin A (CsA) blocked HBV infection. (A) Schematic representation of the schedule for exposing HepaRG cells to compounds and HBV. HepaRG cells were pretreated with compounds for 2 h and then inoculated with HBV for 16 h. After washing out the free HBV and compounds, the cells were cultured with the medium in the absence of compounds for an additional 12 days to quantify HBs protein secreted from the infected cells into the medium. Black and dotted bars indicate the interval for treatment and without treatment, respectively. (B) CsA 4 μM, heparin 25 U/ml, bafilomycin A1 200 nM, lamivudine 1 μM, anti-FLAG 10 μg/ml, and anti-HBs antibody 10 μg/ml, were tested for effect on HBV infection according to the protocol shown in (A). (C-E) HBc protein (C), HBV DNAs, and cccDNA (D) in the cells as well as HBe antigen in the medium (E) at 12 days postinfection according to the protocol shown in (A) were detected by immunofluorescence, real time PCR analysis, southern blot, and ELISA. Red and blue in (C) show the detection of HBc protein and nuclear staining, respectively. (F) Primary human hepatocytes were treated with the indicated compounds and infected with HBV using the protocol shown in (A). The levels of HBV DNAs in the cells, as well as of HBs and HBe antigens in the medium, were determined. Statistical significance was determined using the Student's t-test (*$P<0.05$, **$P<0.01$).

Fig. 2. CsA reduced internalized HBV. (A) HepaRG cells were treated with or without various concentrations of CsA (0.5, 1, 2, and 4 μM) as shown in Fig. 1A. HBV infection was monitored by HBs and HBe secretion. (B) HBV DNA in core particles was detected by southern blot analysis of DNA extracts from HepAD38 cells treated for 6 days with or without tetracycline 0.5 μg/ml and lamivudine 1 μM. (C) Upper scheme indicates the treatment schedule of HepaRG cells with compounds and HBV. HepaRG cells were infected with HBV for 16 h. After washing out the input virus, cells were cultured in the absence of compounds for 8 days. The cells were then cultured with compounds (lamivudine 1 μM, entecavir 1 μM, IFNα 100 IU/ml, or heparin 25 U/ml) for 4 days and recovered for detection of HBV DNA. Black and dotted boxes indicate the periods with and without treatment, respectively. Lower graph shows the quantified relative HBV DNA level in cells treated according to the above scheme. (D) Upper scheme shows the experimental procedure for examining the attached and internalized HBV. (a) The cells were pretreated with compounds

(heparin 25 U/ml, lamivudine 1 μM, or CsA 4 μM) at 4°C for 2 h and then treated together with HBV at 4°C for 3 h for allowing HBV attachment to the cells. After washing out the free virus, cell surface HBV DNA was extracted and quantified by real time PCR. (b) After attachment of HBV at 4°C for 3 h and the following wash, the cells were cultured in the presence or absence of compounds at 37°C for 16 h to allow the cells to internalize bound HBV. The cells were then trypsinized and extensively washed prior to quantifying the cellular HBV DNA. The lower graphs show the level of HBV DNA attached to the cells (a) and internalized inside the cells (b). "Background" in (b) indicates the signal from cells incubated at 4°C, instead of 37°C, for 16 h after washing out the virus in (b), which shows the background signal level of the assay. (E) The upper scheme shows the procedure for the time of addition experiment. Compounds (CsA 4 μM, anti-HBs antibody 10 μg/ml, or heparin 25 U/ml) were applied beginning 2 h prior to HBV infection (a), beginning during HBV infection (b), or beginning immediately after HBV infection (c) until 24 h postinfection. HBs protein secretion was measured at 12 days postinfection. Middle and lower graphs indicate HBs and HBe secretion, respectively, from the cells treated according to the above scheme. (F) Preincubation of HBV with compounds. HBV was preincubated with the indicated compounds for 30 min at 37°C. Compounds were then removed by ultrafiltration. The recovered compound-treated HBV was used to infect HepaRG cells (16 h incubation), and HBV infection was monitored with HBs antigen secreted into the medium at 12 days postinfection. *P<0.05, **P<0.01, N.S. not significant.

Fig. 3. CsA showed a pan-genotypic anti-HBV effect. (A) Primary human hepatocytes were treated with compounds (CsA 4 μM or heparin 25 U/ml) according to the scheme in Fig. 1A with different genotypes of HBV inoculum, and either HBs protein in the medium or HBV DNA in the cells at 12 days postinfection was quantified. (B) CsA did not affect the entry of HCV. Huh-7.5.1 cells were pretreated with the indicated compounds for 1 h and then infected with HCV pseudoparticles (HCVpp) for 4 h. At 72 h postinfection, intracellular luciferase activity was measured. *P<0.05, **P<0.01, N.S. not significant.

Fig. 4. Effect of immunosuppressants on HBV infection. (A, C) HepaRG cells were treated with or without the indicated compounds at 2 μM (FK506 4 μM) in (A), and CsA (2

and 4 μM) and PSC833 (2 and 4 μM) in (C), according to the scheme in Fig. 1A. HBs (A and C) and HBe (C) secretion was determined. (B) Effect of compounds on the activity of calcineurin/NF-AT pathway. Jurkat cells transfected with pNF-AT-luc and pRL-TK were stimulated with PMA and ionomycin in the presence or absence of CsA, FK506, and PSC833 for 24 h to measure the luciferase activity. (D) Cyclophilin binding activity of CsA, FK506, and PSC833 was determined in a competitive binding assay as described in Experimental Procedures, using a CsA-derived fluorescent probe. IC_{50}s (μM) for the inhibition of probe binding to CyPA, CyPB, and CyPD are shown. *P<0.05, **P<0.01.

Fig. 5. NTCP inhibitors blocked HBV infection. (A) NTCP transporter activity was examined following CsA, FK506, rapamycin, and PSC833 treatment of 293 cells overexpressing NTCP, as described in Experimental Procedures. Dose response curves and IC_{50}s for inhibition of NTCP transporter activity are shown. (B) NTCP transporter activity was measured in HepG2-NTCP cells treated with or without CsA 10 μM or tauroursodeoxycholic acid (TUDCA) 10 μM as a positive control. (C) Expression of mRNAs for NTCP, CyPA, CyPB, and GAPDH in HepaRG, primary human hepatocytes, HepG2, Huh-7, HeLa, and FLC4 cells was determined by RT-PCR. (D) HepaRG cells were treated with or without CsA 4 μM, ursodeoxycholate 100 μM, cholic acid 100 μM, propranolol 100 μM, progesterone 40 μM, bosentan 100 μM, and heparin 25 U/ml according to the scheme in Fig. 1A. Secretion of HBs and HBe was quantified. (E) HepG2 cells overexpressing NTCP (HepG2-NTCP) and the parental HepG2 cells were pretreated with or without CsA or heparin for 2 h, and then treated with HBV for 16 h. HBV infection was monitored with HBs and HBe secreted from the cells. (F) AlphaScreen assay to evaluate the binding between NTCP and large envelope protein (LHBs) as described in Experimental Procedures. (a) Left, His-tagged GST (white bars) or NTCP (black bars) were incubated with large (LHBs), middle (MHBs), or small envelope protein (SHBs). Right, His-tagged NTCP and other non-relevant proteins, LCK and FYN, and GST were incubated with LHBs, MHBs, and GST. (b-e) His-tagged GST (white bars) or NTCP (black bars) were incubated with LHBs in the presence of varying amount of pre-S1 lipopeptide HBVpreS/2-48^{myr} (b; 0, 7.7, 15.3, 30.7, and 61.3 μM), CsA (c; 0, 37.5, 75, 150, and 300 μM), FK506 (d; 31, 63, 125, 250, and 500 μM), and SCYX1454139 (e; 0, 37.5, 75, 150, and 300 μM), respectively.

*P<0.05, **P<0.01.

Fig. 6. Analysis of CsA analogs. (A, B) Anti-HBV activity of CsA analogs. HepaRG cells were treated with or without DMSO, heparin 10 U/ml, lamivudine 1 μM, CsA 4 μM, or its analogs, SCYX618806, SCYX1774198, SCYX827830, and SCYX1454139 (A) or alisporivir (B) at 4 μM, as shown in Fig. 1A to measure HBs and HBe secretion level. (C) Chemical structures of CsA and its derivatives. (D) Dose response curves for CsA analogs. HepaRG cells were treated with or without various concentrations of SCYX618806, SCYX827830, or SCYX1454139 (0.25, 0.5, 1, 2, and 4 μM) as shown in Fig. 1A. (E) IC_{50}s (μM) for CsA and its analogs in blocking HBV infection are shown. CC_{50}s (μM) determined by the MTT cell viability assay are also shown. (F) Primary human hepatocytes were treated with CsA and its derivatives at 4 μM or left untreated according to the protocol in Fig. 1A, and HBV infection was monitored by HBs protein secretion. *P<0.05, **P<0.01.

Fig. 1.

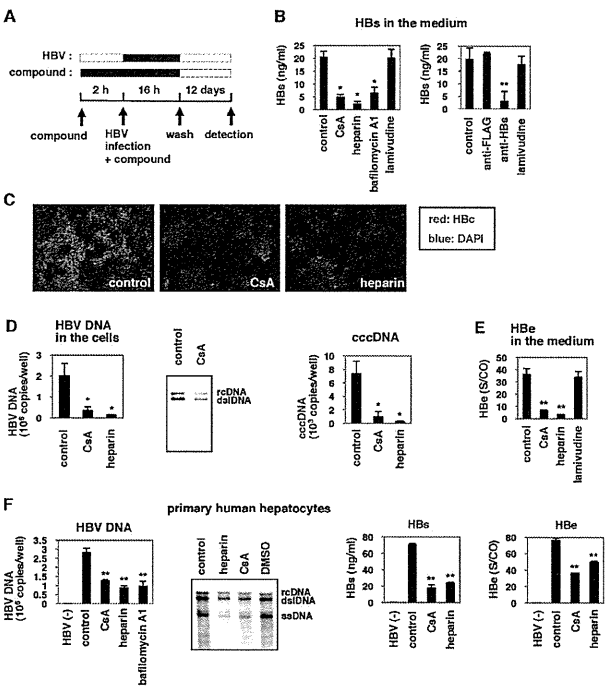

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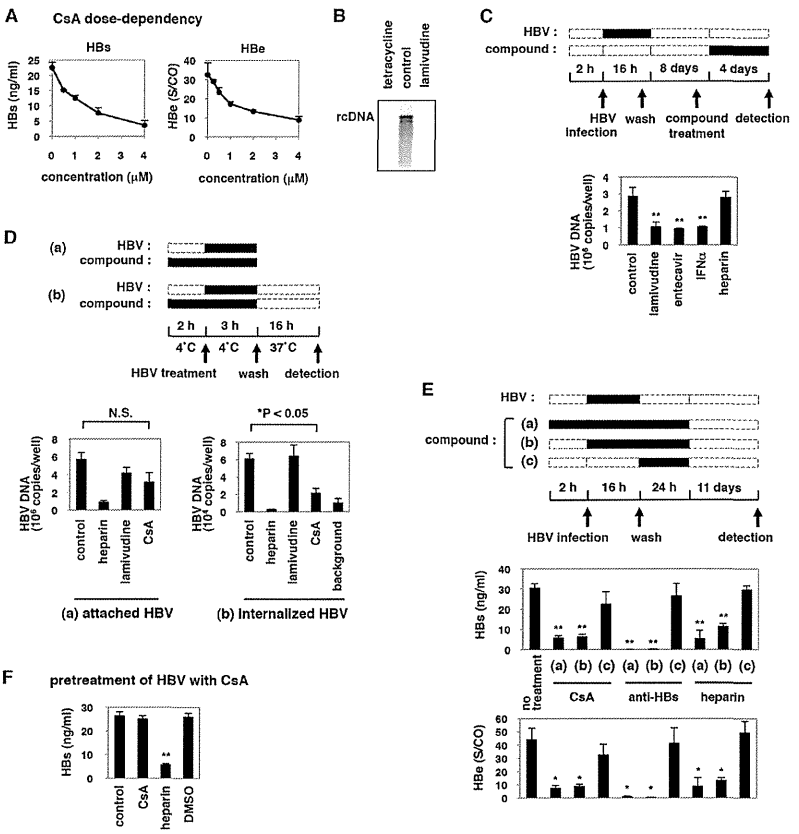
Fig. 2.



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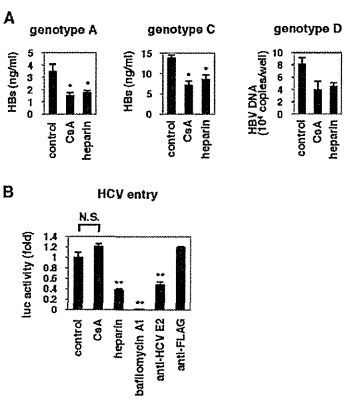

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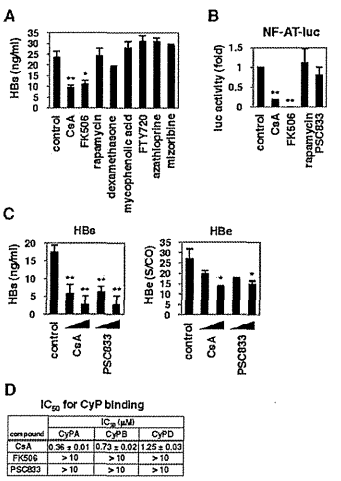

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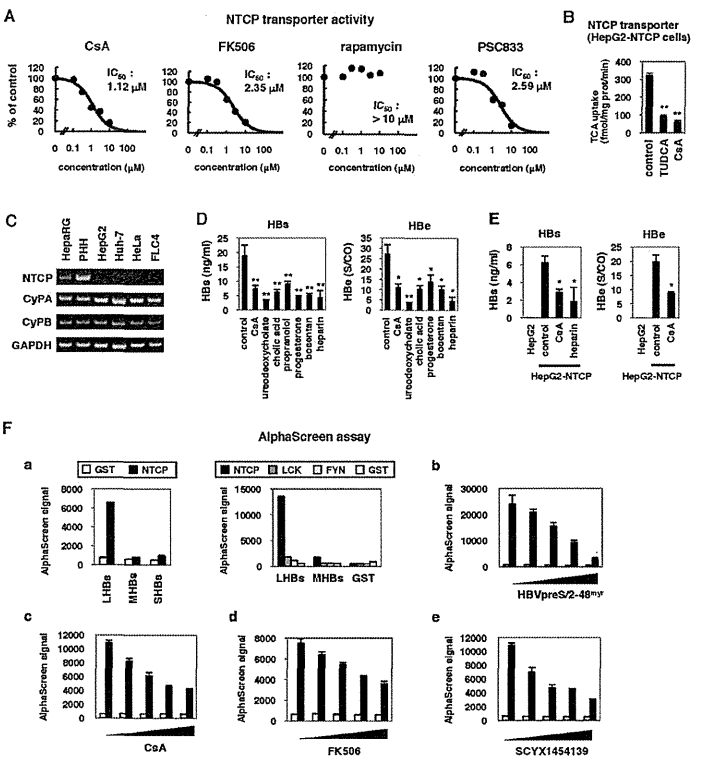

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