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Inhibition of duck hepatitis B virus replication by hypericin

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Summary

Hypericin was found to be active against a member of the hepatitis B virus family, duck hepatitis B virus (DHBV). After a single 1 h incubation with hypericin, cells stably-transfected with a clone of DHBV stopped producing infectious virus for several days, though virus-like particles continued to be released into the culture medium. Characterization of these virions revealed a buoyant density characteristic of infectious virus preparations and lower than that of virus cores, suggesting that the particles were enveloped. Western blot analysis suggested, however, that the viral preS protein in surface antigen particles and, by inference, in virions, was present in covalently cross-linked aggregates. Evidence of a similar level of aggregation of the core subunit of virion nucleocapsids was not found, nor was there evidence of a similar high level of aggregation of cell-associated core and preS proteins. Hypericin was only slightly virucidal against DHBV and culture medium from treated cultures did not block initiation of infection when added to DHBV susceptible cultures prior to a challenge with infectious DHBV. Thus, the primary antiviral activity of hypericin against DHBV replication appears to be exerted at a late step in viral morphogenesis.

Duck Hepatitis B virus; Hypericin; Hepatocyte

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Introduction

Hepadnaviruses can cause both transient and chronic infections in their natural hosts. Chronic infections are generally associated with virus replication in the liver and the presence of virus in the blood stream. Transient infections initially appear similar, but the eventual development of a humoral immune response to the virus envelope is associated with the clearance of the virus from the blood and of infected hepatocytes from the liver. In contrast, circulating antibodies reactive to the virus envelope (i.e., neutralizing antibodies) are virtually never detected in chronically infected individuals. The strong correlation between production of neutralizing antibodies and the clearance of virus from the liver makes it difficult, however, to know if extracellular spread of virus plays an important role in the maintenance of a chronic infection. For example, infected hepatocytes may be slowly killed by the host immune response and replaced by division of uninfected progenitors, which only become susceptible to infection as they terminally differentiate (London and Blumberg, 1982). According to this scenario, blocking by neutralizing antibodies of the recruitment of these cells into the infected cell pool would gradually lead to a virus-free liver, even in the absence of immune-mediated destruction of infected hepatocytes. Immune-mediated destruction of infected hepatocytes is probably seldom active enough to rapidly and completely clear an infection in the presence of circulating virus. Rare cases of fulminant hepatitis in which nearly the entire liver is destroyed after exposure to hepatitis B virus may represent an exception to this rule.

These considerations raise the hypothesis that any agent that blocked extracellular spread of a Hepadnavirus might be used for long-term antiviral therapy leading to the elimination of a chronic infection. Recent studies have suggested that hypericin may represent such an antiviral agent. Several groups have presented evidence suggesting that this compound is either virucidal against enveloped viruses, in vitro, including HIV, herpes simplex, influenza A, murine leukemia virus, murine cytomegalovirus, pseudorabies, and varicella-zoster virus (Tang et al., 1990; Hudson et al., 1991; Lopez-Bazzochi et al., 1991; Sydiskis et al., 1991) and/or that hypericin may inhibit enveloped virus replication in cell culture (Meruelo et al., 1988; Schinazi et al., 1991). Moreover, hypericin has been reported to inhibit murine leukemia virus induced leukemogenesis in infected mice (Meruelo et al., 1988; Lavie et al., 1989), suggesting that the virucidal activity and/or the effect on virus replication is effective in vivo. The present study was carried out to determine if hypericin was active in cell culture against duck hepatitis B virus (DHBV), a member of the Hepadnaviridae family (Mason et al., 1980). We report that the compound inhibited a late step in DHBV maturation, and did not appear to be strongly virucidal.

Materials and Methods

Cells and viruses

Duck hepatitis B virus was obtained from viremic, chronically infected Pekin ducks and from the D2 cell line. The D2 cell line was derived by stable transfection of the chicken hepatoma cell line, LMH (Kawaguchi et al., 1987; Condreay et al., 1990) with an expression vector directing synthesis of the viral pregenome from the CMV immediate-early promotor (Condreay et al., 1990) and of Tn5neo^R from a β-globin promoter. Stable transfectants were selected by growth in medium containing geneticin (G418). The cells were propagated at 37°C in DMEM-F12 medium containing 10% fetal calf serum (Condreay et al., 1990) and supplemented with 100 μg of G418 per ml. G418 was omitted during experimental protocols. The D2 line contained ca. 10–50 copies per cell of viral CCC DNA, which probably serves as the major template for transcription of viral RNAs. Cell culture fluids contained up to 10^9 virons per ml, ca. 20% the level found in duck sera (Jilbert et al., 1992). A more detailed description of this cell line will be presented elsewhere (Jilbert et al., in preparation).

Primary cultures of duck hepatocytes susceptible to DHBV infection were prepared from 2-week old Pekin ducklings free of DHBV-infection and maintained in serum-free L15 medium, following published procedures (Tuttleman et al., 1986; Pugh and Summers, 1989). DHBV infection was carried out 1 day later by addition to the hepatocyte medium of DHBV-positive duck serum or clarified culture fluids from the D2 cell line. Suramin (100 μg per ml) was added to the culture medium as indicated in the text beginning at 1 day post-infection to prevent cell to-cell spread of virus (Petcu et al., 1988). At 3 and/or 8 days post-infection, the hepatocyte monolayers were rinsed with PBS (150 mM NaCl, 7.2 mM Na_2HPO_4, 2.8 mM K H_2PO_4) and stored at −80°C for later DNA extraction. The presence of infectious virus in the inocula was detected by assaying for replicative forms of viral DNA in the hepatocytes.

Analysis of viral DNAs

Total nucleic acids were extracted from primary hepatocyte cultures and subjected to gel electrophoresis in 1.5% agarose, followed by filter blot hybridization, as described by Condreay et al. (1990). Except as indicated, viral DNA present in particles released into D2 culture fluids was extracted, following particle purification by isopycnic centrifugation in sucrose density gradients, essentially as described (Mason et al., 1980). Aliquots of the gradient fractions were digested with SDS-pronase and layered directly onto agarose gels for electrophoresis and blot hybridization. DHBV DNA was detected using a ^{32}P-radiolabeled probe (ca. 10^9cpm per μg) prepared from a DNA clone of the complete viral genome.

Endogenous DNA polymerase reactions were carried out with viral particles pelleted through 10–20% sucrose step gradients and viral DNA radiolabeled in the endogenous reaction was detected by autoradiography following agarose

gel electrophoresis (Mason et al., 1980).

Protein blotting

The immunologic detection of the DHBV preS and core proteins following SDS polyacrylamide gel electrophoresis was done as described (Wu et al., 1991) using rabbit antibodies raised to virus particles purified from duck sera and viral nucleocapsids isolated from infected duck livers, respectively, (Halpern et al., 1984; Pugh et al., 1987; Wu et al., 1991). Cytoplasmic extracts of D2 cells were prepared for electrophoresis as previously reported (Wu et al., 1991). Virus particles were concentrated from culture fluids as described above and in the figure legends and disrupted in electrophoresis sample buffer (Laemmli, 1970).

Materials

Hypericin was prepared from St. Johnswort herb powder as reported previously (Tang et al., 1990). Except as noted, the drug was utilized at a concentration of 10 μg per ml. Experimental manipulations with this compound were carried out in reduced light and treated cells were incubated in the dark.

Results

A brief treatment of D2 cells with hypericin blocks subsequent production of infectious DHBV. Previous work with hypericin suggests that even a brief treatment of infected cells with the compound may cause a prolonged inhibition of infectious retrovirus production (Meruelo et al., 1988; Lavie et al., 1989). To determine the effect of hypericin on DHBV replication, D2 cells producing DHBV were incubated for 1 hour in growth medium containing hypericin, rinsed several times, and then incubated with daily media changes. The culture fluids obtained at various times after treatment were clarified and infectivity was subsequently assayed in primary duck hepatocyte cultures. Treatment with 10 or 1 μg of hypericin per ml blocked infectious virus production by D2 cells for 5 days or more, whereas 0.1 μg per ml was ineffective. No effect on intracellular levels of viral RC-DNA were detected through the same time-period. Hypericin at 100 μg per ml was toxic to the cells. Pseudohypericin, reported to be almost as effective as hypericin against murine leukemia viruses (Meruelo et al., 1988; Lavie et al., 1989), was ineffective against DHBV (not shown). Fig. 1 shows the results of treating D2 cells with 10 μg per ml of hypericin. Virus infectious for primary duck hepatocyte cultures could not be detected until 8 days post-treatment, in contrast to untreated D2 cells, which continuously shed infectious DHBV.

In order to determine if hypericin blocked particle release, virus DNA in viral particles was extracted from D2 culture fluids at various times after treatment with hypericin (10 μg per ml) and subjected to a blot hybridization

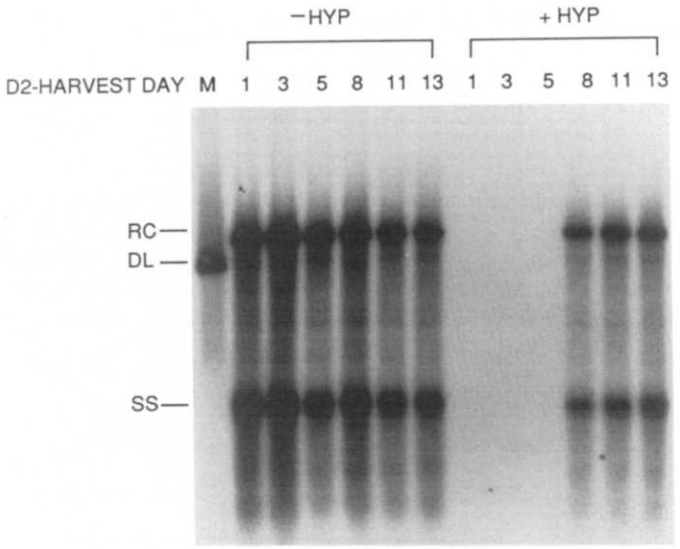

Fig. 1. Hypericin treatment blocks production of infectious DHBV by the D2 cell line. D2 cells were seeded at 3.2×10^6 cells per 60 mm diam. tissue culture dish, medium changed daily, and after 3 days treated for 1 hour with medium containing 10 μg of hypericin per ml. The cultures were then rinsed and, again, medium changed daily. The cells were subcultured once (1:2) at 3 days post-treatment. Untreated controls were done in parallel. Culture medium collected at the indicated number of days post-treatment was tested for infectious virus production by inoculation of 500 μl onto primary duck hepatocyte cultures. Suramin (100 μg/ml; Petcu et al., 1988) was added to the hepatocyte culture medium beginning at 1 day post-infection and medium was changed daily. Total nucleic acids were extracted from the hepatocytes at 8 days post-infection and one quarter of each was subjected to Southern blot analysis. RC, 3 kbp relaxed-circular DHBV DNA; DL, 3 kbp double stranded, linear DHBV DNA; SS, 3 kb, negative strand, DHBV DNA.

assay. As shown in Fig. 2A, hypericin did not have a major effect on the level of viral DNA containing particles in the D2 culture fluids even at times when infectious virus could not be detected (Fig. 2B).

Visual inspection of the D2 cultures treated with hypericin revealed that some of the reddish-colored drug remained associated with the monolayers for several days, which would explain its long-lasting antiviral effect. This also raised the possibility that the antiviral activity might have been mediated by transfer of residual drug or drug metabolite to the primary hepatocyte cultures used to assay infectivity; e.g., that hypericin blocked hepatocyte infection. To test this possibility, hepatocytes were simultaneously co-infected with medium harvested at 3 days post-treatment from hypericin-treated D2 cultures and untreated controls run in parallel, or with either harvest alone. No virus replication was detected in hepatocyte cultures inoculated with medium from hypericin-treated cells. However, equal levels of DHBV DNA replication were detected following infection with culture medium from untreated D2 cells and after co-infection with medium from control and treated cells (not shown). Thus, the antiviral activity of hypericin illustrated in Figs. 1 and 2 does not appear to be due to transfer of hypericin or a metabolite from the D2 to the

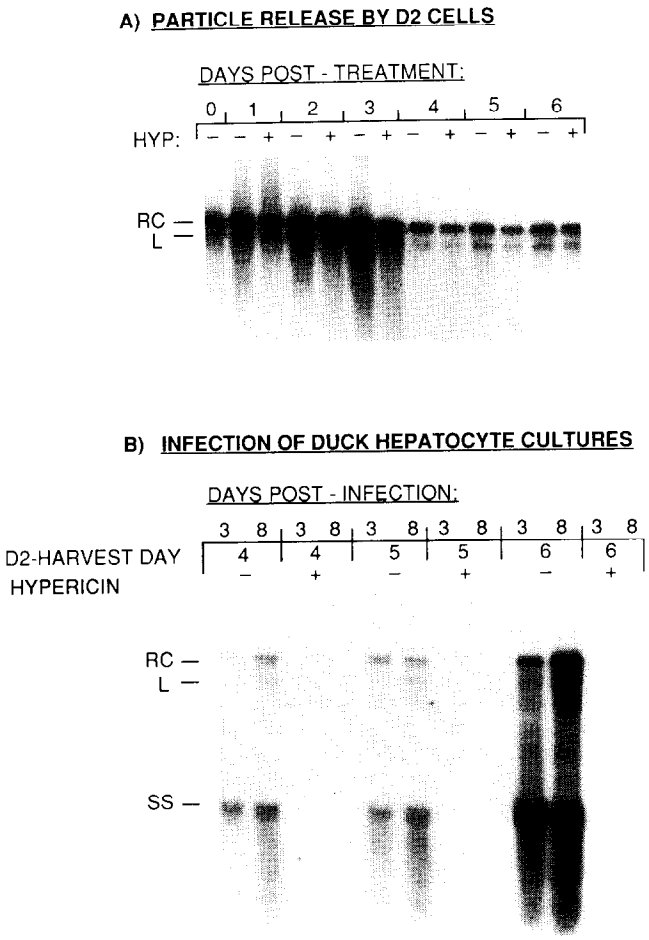

Fig. 2. Hypericin treatment does not prevent the release from D2 cells of particles containing DHBV DNA. D2 cells were grown and treated with 10 μg of hypericin per ml, as described in the legend to Fig. 1. Untreated controls were done in parallel. The D2 cells were subcultured once (1:2) at 3 days post-treatment. (A) Culture medium (400 μl) was clarified and viral DNA isolated by phenol extraction following incubation with SDS-pronase. The viral DNA was detected by Southern blot analysis. (B) Primary hepatocyte cultures were used to assay for virus infectivity following inoculation with 500 μl of culture medium from the D2 cells.

primary hepatocyte cultures.

Hypericin treatment effects the structure of viral particles produced by D2 cultures. Since the antiviral activity of hypericin on DHBV produced in D2 cultures was not due to an effect of the drug on the hepatocytes used to assess viral infectivity, we looked for a direct effect on virus particles. Three variables were considered: particle density, protein composition, and endogenous DNA polymerase activity. Fig. 3 shows that the density distribution of viral DNA

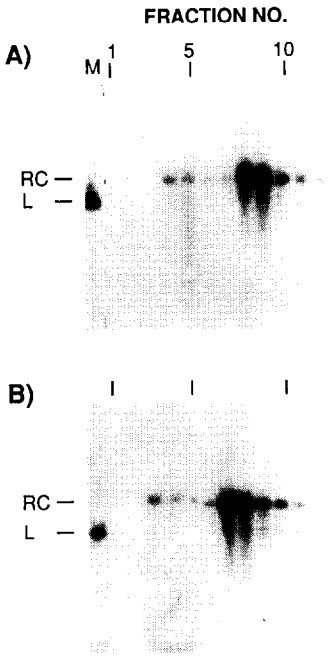

Fig. 3. Virus-like particles are produced by hypericin-treated cultures. At 1 day post-plating, D2 cells were treated with 10 μg of hypericin per ml for 1 hour or left untreated. Culture fluids were harvested at 3 days post-treatment for subsequent analyses. No infectious particles were detected in medium from treated cultures. The culture fluids from the control cells contained ca. 50% more virus particles than the fluids from the treated cells (not shown). To determine the density of virus particles released by the D2 cultures, 3.5 ml culture fluid was layered onto linear, 7 ml 20–60% (w/v) sucrose density gradients containing 0.15 M NaCl, 0.02 M TRIS-HCl (pH 7.5), in H_2O and formed over a 1 ml cushion of 70% (w/v) sucrose, 0.15 M NaCl, 0.02 M TRIS-HCl in D_2O. The gradients were centrifuged in a Beckman SW41 rotor for 17 h at 24000 rpm and 4°C. Fractions (ca. 0.5 ml) were collected from the bottom and the refractive index of each was determined for subsequent estimates of virus density in sucrose. To equal aliquots of each fraction was added 1 mg of pronase per ml and 0.1% (w/v) SDS. After a 1 hour incubation at 37°C the samples were subjected to agarose gel electrophoresis and Southern blot analysis. (A) Treated. (B) Not treated. Density across gradient A ranged from ca. 1.23 (fraction 3) to 1.10 (fraction 12) and across gradient B, from ca. 1.05 to 1.22 g/cc. The cushions had a density of ca. 1.34' g/cc.

containing particles released into the D2 culture medium did not differ significantly between untreated and hypericin-treated monolayers. In either case, particles banded at a density of ca. 1.15 g/cc. These results imply that hypericin does not prevent formation of enveloped virus. We therefore examined the released particles for preS envelope protein content and for viral core (nucleocapsid subunit) antigen.

As shown in Fig. 4, virus harvested from culture fluids of control and hypericin treated D2 cells contained a single major electrophoretic form of nucleocapsid antigen, as previously described by Pugh et al. (1990). In contrast, the major electrophoretic forms of preS (Yokosuka et al., 1989; Schodel et al., 1991) were missing from viral particles released by the hypericin-treated cells. A

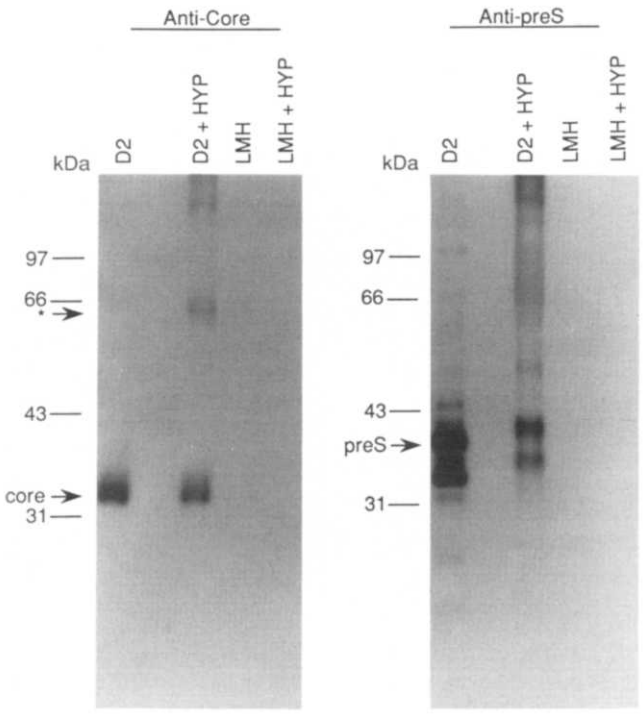

Fig. 4. Hypericin treatment alters DHBV envelope protein in particles released from D2 cells. D2 cells on 100 mm diam. tissue culture dishes were treated for 1 hour with 10 μg of hypericin per ml, as described in the legend to Fig. 1. At 2 days post-treatment, 5 ml of culture fluid was harvested, clarified and virus particles were collected by centrifugation through a 10–20% sucrose step gradient, essentially as described (Mason et al., 1980). Culture fluids from untreated D2 cells and from treated and untreated LMH cells were processed in parallel. For Western blot analyses, the pellets were resuspended, divided into two equal parts, and subjected to SDS polyacrylamide slab gel electrophoresis. Viral proteins were detected with antibodies reactive to nucleocapsid and preS envelope protein, respectively. The position of protein molecular weight standards is indicated at the left side of each panel, as is the position of the core protein subunit of the viral nucleocapsid and the largest preS protein. The asterisk denotes the position of a possible dimer of core proteins in particles from the hypericin-treated culture.

similar deficit was apparent in viral particles banding in fractions 8 to 9 of Fig. 3A, but not 3B (data not shown). Two electrophoretically distinct species with slightly lower electrophoretic mobility were detected (Fig. 4B), and could represent variants of the two species found in the untreated cultures. India ink-staining of the nitrocellulose filters to detect major species of proteins in the different preparations did not reveal a general shift in mobility as a result of the treatment with hypericin, though a band corresponding in position to the larger preS species in the control was missing or greatly reduced in amount after hypericin treatment. However, both the anti-core and the anti-preS sera detected, in Western blots of particles released by cultures that had been treated with hypericin, an excess of slowly migrating proteins that may be cellular in origin, or dimers and more highly aggregated forms of core and preS proteins,

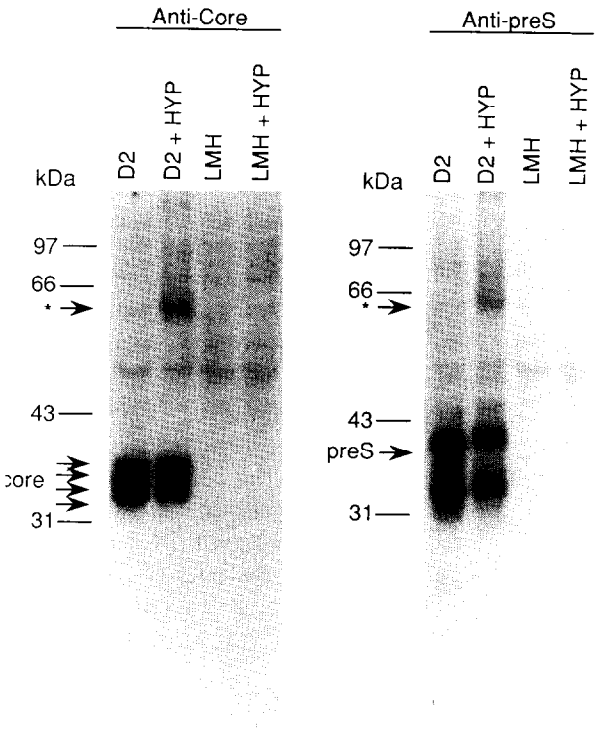

Fig. 5. Viral envelope protein is detectable in D2 cells treated with hypericin. Cytoplasmic proteins were harvested at 2 days post-treatment from the cultures described in the legend to Fig. 4. The monolayers were rinsed with PBS, frozen and thawed, and resuspended in 1 ml per dish of 0.15 M NaCl, 0.01 M TRIS-HCl, (pH 7.4), 0.5% (v/v) nonidet *P*-40, 0.2 Trypsin inhibition units of aprotinin per ml. The cell lysates were clarified for 5′ at 12000 × *g* in a microfuge and the proteins were ethanol precipitated and resuspended in SDS sample buffer (Laemmli, 1970) for SDS polyacrylamide gel electrophoresis and Western blot analysis. One-eighth of a sample was loaded into each lane of the gel. The four arrows next to core in the left hand panel indicate the four major electrophoretic forms of this phosphoprotein that are detected in cytoplasmic extracts (Pugh et al., 1990).

respectively. Our results argue that hypericin either prevented incorporation of preS protein into viral particles or induced an alteration in its electrophoretic behavior. Because DHBV produces a vast excess of noninfectious 'surface antigen' particles as compared to virus (Mason et al., 1980), we conclude that the preS protein in surface antigen particles was affected by hypericin. We infer that preS of virus was similarly affected.

To determine the effect of hypericin upon cell-associated viral proteins, cell extracts were also subjected to Western blot analyses (Fig. 5). However, no significant shift in electrophoretic mobility of the two major preS species and the multiple electrophoretic variants of intracellular core antigen (Pugh et al., 1990) were now detected. The slight shift seen in lanes two (D2 + HYP) and six

(D2 + HYP) was also apparent with the india ink-stained cellular proteins and may reflect a difference in viscosity of the samples, as it was not observed in other preparations. Again, additional slowly migrating antigens, including a prominent band at ca. 60–70 kdal, were detected by the antisera in the hypericin treated samples (cf, Fig. 4).

Taken together, the data suggest that hypericin either prevents incorporation of preS into virus particles or causes specific incorporation of a variant preS protein. The results in Fig. 4 are also consistent with the idea that extracellular viral particles contained some altered, perhaps dimerized or more highly aggregated viral core protein. The endogenous DNA polymerase reaction of extracellular DNA-containing viral particles from cells treated with 10 μg of hypericin per ml also appeared to be slightly reduced compared to the control (data not shown). Though approximately the same amount of viral DNA was detected in the two viral particle preparations, ca. 3-fold less incorporation of 32P-TTP into DNA was observed in a standard 1 hour reaction at 37°C (Mason et al., 1980). This result supports the conclusion that hypericin at high concentration (10 μg per ml) can alter components of the virus nucleocapsid required for DNA polymerization. The effect on the polymerase reactions may not be a useful indicator of the antiviral reactivity of hypericin, however, since hypericin at 1 μg per ml was active against infectious virus production but endogenous DNA polymerase activity did not appear to be inhibited.

Hypericin is weakly virucidal against DHBV. Hypericin is virucidal against a variety of enveloped viruses (Tang et al., 1990). To assay for activity against DHBV, 50 μl of DHBV positive medium from D2 cells buffered with 0.01 HEPES (pH 7.5), was incubated 1 hour at 37°C in the presence or absence of 10 μg of hypericin per ml. Surviving infectivity was assayed on primary hepatocyte cultures. Incubation at 37°C resulted in a ca. 3-fold inactivation of DHBV and an additional 2-fold inactivation was seen in the presence of hypericin. We have also found that the preS protein was still detected by Western blot analysis of hypericin-treated virus. No significant inactivation of DHBV was observed when 1 μl of DHBV positive serum was mixed with 50 μl of culture medium from hypericin-treated cells, which contained inactivated DHBV, and incubated 1 hour at 37°C in the presence of 0.01 HEPES buffer, pH 7.5 (not shown).

Discussion

Our results demonstrated that hypericin blocks production of infectious DHBV by the D2 cell-line for several days after a brief treatment with the drug. The fact that hypericin was only weakly virucidal argues against the possibility that residual drug released from the treated cells into the culture medium inactivated mature virus to produce the observed effect. Moreover, in a preliminary experiment described above, we were not able to detect any

significant virucidal activity in culture medium from cells that had been previously treated with hypericin. From these observations, we concluded that hypericin is most active at a late step in viral morphogenesis. We can not rule out, however, that the antiviral activity is exerted subsequent to incorporation of drug into otherwise normal virus particles.

Western blot and density gradient analyses of virus particles suggested that hypericin caused the release of DHBV surface antigen and, by inference, virus particles in which the preS proteins are, or became, irreversibly cross-linked. Because of the unavailability of antibodies reactive to the S protein of DHBV (Schodel et al., 1991), we were unable to determine if this envelope component was also effected. Some cross-linking of the core protein, the viral nucleocapsid subunit, was detected (Fig. 4). The fact that hypericin treatment did not significantly reduce the amount of DNA-containing virions released from the D2 cells (Fig. 2) suggested that this cross-linking was not associated with an inhibition of intracellular viral DNA synthesis, which takes place in immature nucleocapsids (Summers and Mason, 1982).

Several questions remain to be answered about the anti-DHBV activity of hypericin. Primary among these is if hypericin is active in vivo, not only against DHBV but also against human hepatitis B virus, and whether the type of activity exhibited by hypericin, inhibition of cell-to-cell spread of virus, would have any effect against a chronic infection. The answer to the first question should be rather straightforward to obtain. If hypericin has good in vivo activity, treatment of viremic hosts should lead to a loss of infectivity of virus released into the circulatory system. The answer to the second question is more difficult to predict. Hypericin does not appear to block intracellular events in virus replication. Moreover, Hepadnavirus infections per se do not appear to be particularly cytotoxic and, for this reason, can ultimately involve essentially the entire hepatocyte population of the liver. If hepatocytes that die spontaneously or as a result of immune responses to viral antigens are normally replaced by division of adjacent, mature hepatocytes, which would probably be infected, then hypericin would not be useful, by itself, as a therapeutic agent, though it might be useful if given together with agents acting at other steps in the virus life-cycle. On the other hand, if dying hepatocytes are replaced by division of uninfected progenitor cells, or if cell division results in a loss of viral DNA from a cell, then hypericin treatment should permit, over time, the replacement of infected by uninfected hepatocytes. This latter scenario might reflect the situation in infected individuals, since transient Hepadnavirus infections involving the majority of hepatocytes do resolve (Barker et al., 1973; Berquist et al., 1975; Hoofnagle et al., 1978; Ponzello et al., 1984; Jilbert et al., 1992) once a host produces virus neutralizing antibodies in excess of virus particles in the serum.

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References

Barker, L.F., Chisari, F.V., McGrath, P.P., Dalgard, D.W., Kirschstein, R.L., Almeida, J.D., Edgington, T.S., Sharp, D.G. and Peterson, M.R. (1973) Transmission of type B viral hepatitis to chimpanzees. J. Infect. Dis. 127, 648–662.

Berquist, K.R., Peterson, J.M., Murphy, B.L., Ebert, J.W., Maynard, J.E. and Purcell, R.H. (1975) Hepatitis B antigens in serum and liver of chimpanzees acutely infected with hepatitis B virus. Infect. Immun. 12, 602–605.

Condreay, L.D., Aldrich, C.E., Coates, L., Mason, W.S. and Wu, T.-T. (1990) Efficient duck hepatitis B virus production by an avian liver tumor cell line. J. Virol. 64, 3249–3258.

Halpern, M.S., England, J.M., Flores, L., Egan, J., Newbold, J. and Mason, W.S. (1984) Individual cells in tissues of DHBV-infected ducks express antigens cross-reactive with those on virus surface antigen particles and immature cores. Virology 137, 408–413.

Hoofnagle, J.H., Michalak, T., Nowoslawski, A., Gerety, R.J. and Barker, L.F. (1978) Immunofluorescence microscopy in experimentally induced, type B hepatitis in the chimpanzee. Gastroenterology 74, 182–187.

Hudson, J.B., Lopez-Bazzochi, I. and Towers, G.H.N. (1991) Antiviral activities of hypericin. Antiviral Res. 15, 101–112.

Jilbert, A.R., Wu, T.-T., England, J.M., De la M. Hall, P., Carp, N.Z., O'Connell, A.P. and Mason, W.S. (1992) Rapid resolution of duck hepatitis B virus infections occur after massive hepatocellular involvement. J. Virol. 66, 1377–1388.

Kawaguchi, T., Nomura, K., Hirayama, Y. and Kitagawa, T. (1987) Establishment and characterization of a chicken hepatocellular carcinoma cell line, LMH. Cancer Res. 47, 4460–4464.

Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680–682.

London, W.T. and Blumberg, B.S. (1982) A cellular model of the role of hepatitis B virus in the pathogenesis of primary hepatocellular carcinoma. Hepatology 2, 10S–14S.

Lopez-Bazzochi, I., Hudson, J.B. and Towers, G.H.N. (1991) Antiviral activity of the photoactive plant pigment hypericin. Photochem. Photobiol. 54, 95–98.

Mason, W.S., Seal, G. and Summers, J. (1980) Virus of Pekin ducks with structural and biological relatedness to human hepatitis B virus. J. Virol. 36, 829–836.

Meruelo, D., Lavie, G. and Lavie, D. (1988) Therapeutic agents with dramatic antiretroviral activity and little toxicity at effective doses: aromatic polycyclic diones hypericin and pseudohypericin. Proc. Natl. Acad. Sci. USA 85, 5230–5234.

Petcu, D.J., Aldrich, C.E., Coates, L., Taylor, J.M. and Mason, W.S. (1988) Suramin inhibits in vitro infection by duck hepatitis B virus, Rous sarcoma virus, and hepatitis delta virus. Virology 167, 385–392.

Ponzetto, A., Cote, P.J., Ford, E.C., Purcell, R.H. and Gerin, J.L. (1984) Core antigen and antibody in woodchucks after infection with woodchuck hepatitis virus. J. Virol. 52, 70–76.

Pugh, J.D., Sninsky, J.J., Summers, J.W. and Schaeffer, E. (1987) Characterization of a preS polypeptide on the surfaces of infectious avian Hepadnavirus particles. J. Virol. 61, 1384–1390.

Pugh, J.C. and Summers, J.W. (1989) Infection and uptake of duck hepatitis B virus by duck hepatocytes maintained in the presence of dimethyl sulfate. Virology 172, 564–572.

Pugh, J.C., Zweidler, A. and Summers, J.W. (1990) Characterization of the major duck hepatitis B virus core particle protein. J. Virol. 63, 1371–1376.

Schinazi, R.F., Chu, C.K., Babu, J.R., Oswald, B.J., Saalman, V., Cannon, D.L., Eriksson, B.F.H. and Nasr, M. (1990) Anthraquinones as a new class of antiviral agents against human immunodeficiency virus. Antiviral Res. 13, 265–272.

Schodel, F., Weimer, T., Fernholz, D., Schneider, R., Sprengel, R., Wildner, G. and Will, H. (1991) The biology of avian hepatitis B viruses. In: A. McLachlan (Ed), Molecular biology of the hepatitis B virus. CRC press, pp. 53–80. Boca Raton, Florida.

Sydiskis, R.J., Owen, D.G., Lohr, J.L., Rosker, K.-H.A. and Blomster, R.N. (1991) Inactivation of enveloped viruses by anthraquinones extracted from plants. Anti. Microb. Agents Chemothr. 35, 2463–2466.

Tang, J., Colacino, J.M., Larsen, S.H. and Spitzer, W. (1990) Virucidal activity of hypericin against enveloped and non-enveloped DNA and RNA viruses. Antiviral Res. 13, 313–326.

Tuttleman, J.S., Pugh, J.C. and Summers, J.W. (1986) In vitro experimental infection of primary duck hepatocyte cultures with duck hepatitis B virus. J. Virol. 58, 17–25.

Wu, T.-T., Condreay, L.D., Coates, L., Aldrich, C. and Mason, W.S. (1991) Evidence that less-than-full-length pol gene products are functional in Hepadnavirus DNA synthesis. J. Virol. 65, 2155–2163.

Yokosuka, O., Omata, M. and Ito, Y. (1989) Expression of pre-S1, pre-S2 and C proteins in duck hepatitis B virus infection. Virology 167, 82–86.