Suramin Prevents Duck Hepatitis B Virus Infection In Vivo

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The effect of suramin on duck hepatitis B virus (DHBV) infection was investigated in vivo. Suramin pretreatment of Pekin ducklings completely prevented DHBV infection. In contrast, suramin given at the time of or after inoculation with DHBV did not inhibit viral infection, replication, or gene expression. These data indicate that suramin effectively blocks the early stages of DHBV infection in vivo.

Suramin, a polysulfonated naphthylurea, was the first clinically useful antiparasitic drug and is still one of the most commonly used antitrypanosomal agents. Apart from its antiparasitic effects, suramin has been demonstrated to have a number of biochemical activities (7). Most notably, suramin has been shown to inhibit a number of enzymes, such as hexokinase, lysosome (11), thrombin, plasma kallikrein (5), and protein kinase C (6). Since suramin was shown to bind to a number of growth factors (8) and protein kinase C is involved in tumor promotion and differentiation, the antitumor activity of suramin has been studied in patients with metastatic adrenocortical carcinoma (18) and metastatic prostate cancer (9). In addition to its antiparasitic and antitumor properties, suramin has been shown to strongly inhibit duck hepatitis B virus (DHBV) DNA polymerase (19, 20) and the reverse transcriptase activities of a number of retroviruses (3), including the human immunodeficiency virus (13). A clinical trial in 10 patients with AIDS demonstrated that suramin indeed inhibits human immunodeficiency virus replication; immunological parameters and clinical outcomes were not affected, however (1).

After the discovery of DHBV (12), Pekin ducks have become a widely used animal model to study hepadnaviral infection. By using this model, antiviral agents can be studied in vitro in replicative complexes isolated from DHBV-infected livers (16) or in primary duck hepatocyte cultures (18) as well as in vivo in DHBV-infected Pekin ducks (15). In primary duck hepatocyte cultures, suramin blocks infection when it is present before and during incubation of the cells with DHBV (17). Since suramin inhibits DHBV infection in vitro, we tested the effect of suramin on DHBV infection in vivo.

Pekin ducks were used as an in vivo model of DHBV infection. One-day-old ducklings were purchased from a German duck farm. Blood was taken from the foot vein and tested for the presence of DHBV DNA by dot blot hybridization (14). DHBV DNA-negative ducklings were infected at the age of 2 or 3 days by intravenous injection of 100 μl of DHBV-positive serum (about 10⁹ virions per ml). Treatment of the animals with suramin (Germanin; Bayer, Leverkusen, Germany) was started before, during, or after DHBV infection of the animals. After 14 days, the ducklings were sacrificed. Livers and sera were stored at −80°C until analysis. The number of animals per experimental group could be kept very low (three ducklings treated with suramin, one nontreated duckling as a negative control) because in our hands DHBV infection of more than 500 ducklings between days 1 and 5 after hatching resulted in a 100% infection rate. The study protocol was approved by the governmental committee on the treatment of laboratory animals.

For Southern blot analysis, DNA was extracted from liver tissues as described previously (15). DNA samples were electrophoresed on a horizontal 1.25% agarose slab gel, denatured, and transferred to Hybond N membranes (Amersham Buchler, Braunschweig, Germany), as recommended by the manufacturer. After prehybridization, the blots were hybridized with a full-length DHBV DNA probe and labeled by nick translation with [32P]dCTP (Amersham Buchler) to a specific activity of about 10⁶ dpm/μg (22).

For Western blot (immunoblot) analyses, serum samples were denatured, subjected to sodium dodecyl sulfate–12% polyacrylamide gel electrophoresis, and transferred to nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany). The membrane was incubated overnight with a polyclonal antibody against DHBV presurface (pre-S) and surface (S) antigens (DHBpre-S/S) raised in rabbits. The following incubations with swine anti-rabbit immunoglobulin, peroxidase-antiperoxidase complex, and substrate solution were carried out according to the instructions of the manufacturer (Dakopatts, Hamburg, Germany). In DHBV-positive control sera, this antibody detected a major protein with a molecular mass of about 36 kDa (pre-S/S protein), some minor species with different molecular masses because of the different degree of glycosylation, and a minor protein with a molecular mass of 18 kDa (S protein).

In a first experiment, three Pekin ducks were treated with suramin prior to infection with DHBV-positive serum; one Pekin duck served as a negative control. Ducklings, weighing about 50 g at hatching and about 150 g after 2 weeks, received 40 mg of suramin per kg of body weight by intravenous injection on days 2, 3, 4, 7, and 10. On day 3, the ducklings were infected by the intravenous injection of 100 μl of DHBV-positive serum. The ducks were sacrificed on day 14. DNA was extracted from the livers and analyzed by Southern blot hybridization as described above. There was no difference in the amount of DNA extracted from the livers of suramin-treated and nontreated animals. As shown in Fig. 1, the untreated control animal (Fig. 1, lane 1) was successfully infected with DHBV, resulting in the presence of various replicative forms of DHBV DNA in the liver, ranging from short single-stranded DNA species of minusstrand polarity to complete double-stranded DNA molecules...
migrating in the 3.0-kbp position. In contrast, in the three animals pretreated with suramin (Fig. 1, lanes 2 to 4), no viral DNA could be detected.

Western blot analyses of serum from the untreated control animal showed the expected pre-S/S and S proteins, demonstrating successful infection of the animal (Fig. 2, lane 1). In contrast, animals pretreated with suramin were negative for DHBpre-S/S antigens in serum (Fig. 2, lanes 2 to 4).

In order to distinguish between a block of DHBV infection and inhibition of DHBV replication after infection, suramin therapy was started at the same time as DHBV infection or 1 day after DHBV infection. Ducklings were infected with DHBV-positive serum on day 3 as described above. Suramin (40 mg/kg) was given on days 3, 4, 5, 8, and 11. The ducks were sacrificed on day 14. The untreated control duck (Fig. 1, lane 5) was again successfully infected. Different from the data obtained after pretreatment of the ducklings with suramin (Fig. 1, lanes 2 to 4), suramin given at the time of inoculation with DHBV-positive serum did not prevent infection but resulted in a productive infection with DHBV DNA replication in the liver (Fig. 1, lanes 6 to 8). Also at the protein level, suramin given at the time of DHBV infection did not block viral infection and resulted in DHBpre-S/S antigen synthesis and export (Fig. 2, lanes 6 to 8) that were indistinguishable from those for the untreated control animal (Fig. 2, lane 5).

In a further experiment, three ducklings were treated with suramin starting 1 day after DHBV infection on day 4, and treatment was continued on days 5, 6, 9, and 12. One duckling served as a negative control. On day 14 the animals were sacrificed and analyzed for DHBV DNA and DHBpre-S/S antigens as described above. As demonstrated in Fig. 1 and 2, respectively, treated (lanes 10 to 12) and untreated (lanes 9) animals showed identical amounts of viral replicative intermediates in liver and viral antigens in serum. Suramin therefore does not affect viral replication and gene expression in DHBV-infected hepatocytes in vivo.

Our data demonstrate that the infection of Pekin ducklings with DHBV can be effectively blocked by pretreatment of the animals with suramin. The dose of suramin used in our in vivo study is about two times higher than that used in vivo studies in humans (1) and ducks (19). Even at this dose, the toxic effects of suramin were not observed during the short-term administration of the drug over 10 to 12 days. Studies to define the minimal dose required for the prevention of DHBV infection and to specifically address the question of toxic side effects are in progress.

Since suramin given at the time of inoculation with DHBV or later does not prevent viral infection, we assume that suramin acts at a very early step of the infection process, such as at the viral attachment or uptake stage. These findings extend the observations of Petcu et al. (17) and ourselves, demonstrating the inhibition of DHBV infection of primary duck hepatocytes in vitro by suramin (Fig. 3). So far, the molecular mode of action of suramin that leads to a block of DHBV infection is not known. It is conceivable that suramin affects endocytosis of DHBV, viral uncoating, or cycling of receptors. Since known inhibitors of viral attachment to target cells, e.g., sulfated polysaccharides such as dextran sulfate or heparin, do not affect DHBV infection in vitro (Fig. 3) or in vivo (14), it is unlikely that suramin acts through this mechanism.
Suramin is concentrated within lysosomes (4). There, it may directly inhibit the lysosomal enzymes important for virus internalization or it may change the lysosomal pH, thereby preventing the fusion of viral and lysosomal membranes. Using lysosomotropic agents, such as ammonium chloride or chloroquine, we previously demonstrated a strong inhibition of DHBV infection in vitro (15). These data indicate that DHBV infection, similar to infections with...
other enveloped viruses, depends on receptor-mediated endocytosis and involves membrane fusion triggered by low pH. In vitro experiments demonstrated that the addition of suramin 2 h after infection and that the addition of lysosomotropic agents 3 h after infection still effectively blocked viral infection (17), suggesting that suramin and lysosomotropic agents have similar mechanisms of action. The fact that in in vivo experiments suramin given at the time of inoculation did not prevent DHBV infection may be due to the pharmacokinetic properties of suramin. In vivo suramin is mostly bound to plasma proteins (99.7%), which may reduce or delay its biological availability (2). By increasing the dose of suramin, this lack of effect of suramin given at the time of infection may be overcome. Furthermore, suramin has been shown to be a P2 purinoceptor agonist and competitively inhibits P2 purinoceptor agonist-induced phospholipase C stimulation (10). It is conceivable that suramin may act through such a mechanism that leads to a block of virus entry.

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