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Selective Inhibition of the Duck Hepatitis B Virus by a New Class of Tetraazamacrocycles

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The antiviral activity of a new class of *N,N,N′,N″,N‴*-pentakis (ω-aminoalkyl) tetraazamacrocycles was evaluated in primary duck hepatocyte cultures infected with the duck hepatitis B virus (DHBV). Three of the four tested compounds were able to selectively inhibit DHBV replication by acting at an early step of the hepadnavirus infection but were associated with significant toxicity.

Chronic infection with hepatitis B virus (HBV) may result in the development of chronic active hepatitis which can lead to cirrhosis and primary hepatocellular carcinoma (3). Today, interferon therapy is the most efficient available treatment (11), but the therapeutical success of interferon therapy is limited and less than 50% of treated patients ultimately enter into remission (10). Promising results have been described with new nucleoside analogs such as famciclovir (13, 24) and lamivudine (2′,3′-dideoxy-3′-thiacytidine [3TC]) (6). However, the decline of HBV viremia is often transient after a short course of famciclovir or lamivudine and long-term therapy may lead to the emergence of resistant virus (16, 22). A search for other effective drugs and new strategies is therefore essential. Such new therapeutic strategies have been extensively investigated for the human immunodeficiency virus (HIV). Recently, a new class of potent and specific inhibitors of HIV types 1 and 2, the bicyclams, has been described (2, 4, 5). These compounds interact with an early step of the virus life cycle, presumably the viral uncoating event.

We have recently studied the anti-HIV activity of several *N,N′,N″,N‴*-tetrakis (ω-aminoalkyl) tetraazamacrocycles. Using the duck hepatitis B virus (DHBV) model (26), we also investigated their antihepadnaviral activity. Unexpectedly, during the synthesis of these new compounds, we also isolated, characterized, and purified as side products the corresponding *N,N,N′,N″,N‴*-pentakis (ω-aminoalkyl) tetraazamacrocycle monoammonium salts which have been tested for their anti-HIV activity (23). Here, we report that these compounds showed significant anti-DHBV activity and inhibit at an early step in the DHBV replicative cycle.

The synthesis and the spectroscopic data of compounds 1a, 1b, 2a, and 2b (Fig. 1) have already been described (23).

The isolation and infection of the hepatocytes were performed as previously described (25). Cells were seeded on six-well plates at a density of 5×10^5 per well in Leibowitz medium supplemented with 5 μg of bovine insulin per ml, 7×10^{-5} M hydrocortisone hemisuccinate, and 10% fetal calf serum. One day after cell plating, the medium was changed with fresh medium without fetal calf serum. The compounds that were tested for antiviral activity were added to the culture medium as indicated below. Medium was changed daily and saved for further testing. Cells were infected with 0.1 ml of DHBV-positive serum (containing approximately 4×10^9 DNA genome equivalents per ml) per well at day 2 postplating for 2 h at 37°C.

DHBV DNA was detected in the hepatocyte culture supernatants (1.5 ml) by a DNA-spot hybridization assay (15). After autoradiography, the individual spots were counted in a scintillation counter. The IC_{50} was defined as the drug concentration that induces a 50% decrease in the level of DHBV DNA in culture supernatants and was calculated by linear regression analysis. Data from three independent experiments were combined.

Protein-bound (viral DNA intermediates) and non-protein-bound (covalently closed circular viral DNA) intracellular DNA were separated by selective precipitation of sodium dodecyl sulfate-complexed protein with KCl (21). Viral DNAs (corresponding to 10 μg of total cellular DNA or 1/10 of the cells in a single well) were separated by 1% agarose gel electrophoresis, transferred to nitrocellulose, and hybridized with a DHBV DNA probe.

Cytotoxicity measurements were based on the estimation of hepatocyte viability after drug treatment by uptake of neutral red dye. Hepatocytes in 24-well tissue culture plates were cultured in medium containing various concentrations of the tested compound with daily changes of medium. Four wells per assay were used. After 9 days of treatment, cell viability was estimated (8). The CC_{50} was defined as the concentration required to reduce cell viability by 50%.

Since we expected that the tetraazamacrocycle derivatives would act at an early step of the virus life cycle, hepatocytes were incubated for 8 h with the tested compounds prior to infection with a standard DHBV inoculum. Treatment was then prolonged for 4 days, and levels of viral replication in treated culture were estimated by quantification of DHBV DNA in cell supernatants. In this short-term anti-DHBV assay, three of the four compounds tested (compounds 1a, 1b, and 2b) were active with an IC_{50} of around 1 μM (Table 1). Compound 1b was the most active against DHBV with an IC_{50} of 0.6 μM. Remarkably, all three compounds active against DHBV were inactive or only slightly active against HIV (23), indicating that they were highly specific. Conversely, com-

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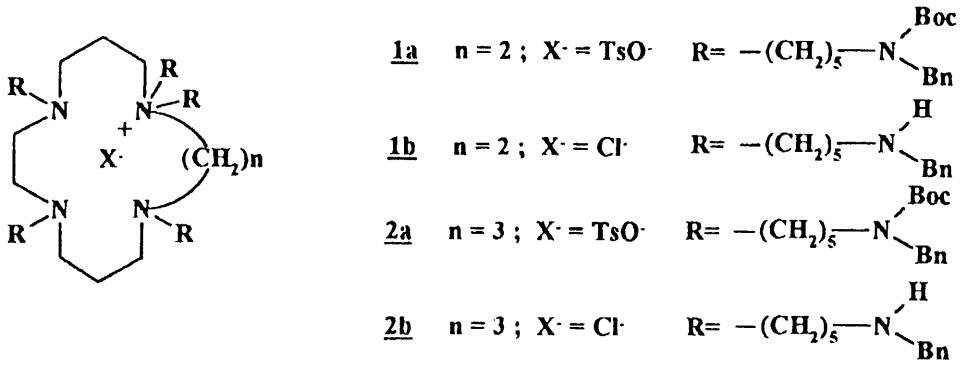

FIG. 1. Schematic representation of *N*,*N*,*N*′,*N*″,*N*‴-pentakis (ω-aminoalkyl) tetraazamacrocycle.

pound 2a, which is inactive against DHBV, inhibited HIV with a 50% inhibitory concentration of 0.1 μM.

Cell viability after long-term treatment indicated that all compounds were significantly toxic. Compound 2b exhibited the greatest toxicity (CC_{50}, 2 μM) while compound 1b was the least toxic (Table 1).

In order to determine at which step of the virus cycle life the compounds act, a time course experiment was performed with compound 1b. A low concentration of 1b (0.5 μM) was used in order to exclude any toxic effect of the compound. The reverse transcriptase inhibitor 3TC (0.2 μM) (7) was used as a control. Hepatocytes were infected, and the compounds were added 8 h before, simultaneously, or 1, 8, or 16 h after infection. Extracellular DHBV DNA obtained 3 days after infection was analyzed by dot blot analysis. Typical results are shown in Fig. 2 and indicate that compound 1b inhibits viral replication when added before or during infection but has only minimal effect when added 16 h after infection. Similar results were obtained with compounds 1a and 2b (data not shown). By contrast, the nucleoside analog 3TC was still effective when added as late as 24 h after infection (Fig. 2).

Analysis of intracellular viral DNA (Fig. 3) confirmed this finding and indicated that compound 1b inhibits formation of relaxed circular and covalently closed circular DNA when added 8 h before but is inactive if added 16 h after infection. By contrast, 3TC was equally effective when added prior to or after infection. This difference between compound 1b and 3TC was also observed when drug treatment was stopped and cell culture was continued for 3 additional days (Fig. 3). 3TC withdrawal was followed by a clear rebound of viral replication as is usually observed. By contrast, cessation of treatment with compound 1b was associated with only a slight increase of DHBV replication.

Few low-molecular-weight inhibitors acting at a step other than those catalyzed by the hepadnaviral polymerases have been described. The imino sugar *N*-butyldeoxynojirimicin, an inhibitor of oligosaccharide trimming, suppressed secretion of HBV particles (1), as did the plant extract glycyrrhizin (20). Suramin is believed to block virus entry (18, 19), while the aromatic polycyclic dion hypericin has only weak virucidal activity against DHBV (17). The results presented here demonstrate that some *N*,*N*′,*N*″,*N*‴-pentakis tetraazamacrocycle derivatives represent a new class of potent inhibitors of DHBV replication in vitro. Three of the four compounds tested were found to be specific inhibitors of DHBV replication and had earlier been found to be poor inhibitors of HIV replication (23). Their precise mode of action remains to be determined, but time course experiments indicate that they are likely to act at an early step of infection between virus penetration and reverse transcription. For HIV, the molecular target of bicyclam seems to be the envelope protein gp120, since bicyclam-resistant viruses harbor mutations exclusively in gp120 (5). By analogy, the compounds described here could interact with the pre-S protein of DHBV. If so, a critical issue is now to determine whether these compounds inhibit replication of other human and woodchuck hepadnaviruses which possess pre-S sequences which differ from those of DHBV.

Unfortunately, these tetraazamacrocycle derivatives are also associated with significant toxicity, which precludes their use for in vivo experiments. Effort must be devoted to designing compounds derived from compound 1b which are less toxic but retain antiviral activity. If confirmed in vivo, the specific antiviral activity of these tetraazamacrocycles may prevent cell-to-cell spread of virus. Whether this type of activity would have any effect on chronic infection is difficult to predict. HBV infection may involve the entire population of hepatocytes (12). If dying hepatocytes are replaced by division of already

TABLE 1. Anti-DHBV properties of new pentakis (5-amino-substituted pentyl) tetraazamacrocycle monoammonium salts[a]

Compound	IC_{50} (μM)	CC_{50} (μM)	SI[b]
1a	1.2 ± 0.1	15 ± 3	12.5
1b	0.6 ± 0.06	10 ± 1	16.6
2a	Inactive[c]	2 ± 0.05	NA
2b	1.0 ± 0.1	6.5 ± 0.7	6.5
3TC	0.2 ± 0.04	450 ± 40	2,250

[a] Data are averages (± standard deviations) of three independent experiments.
[b] SI, selective index (CC_{50}/IC_{50}); NA, not applicable.
[c] Inactive at noncytotoxic concentration (0.1 to 1 μM).

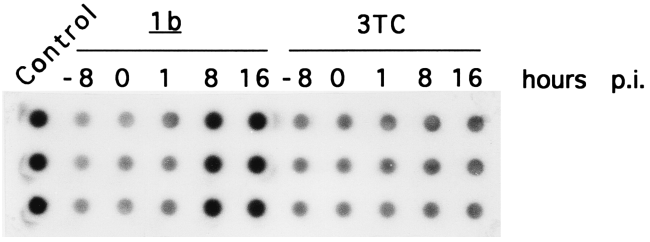

FIG. 2. Dot blot analysis of DHBV DNA in culture supernatant of duck hepatocytes 5 days after in vitro infection. Compounds 1b (0.5 μM) and 3TC (0.2 μM) were added 8 h before or 0, 1, 8, or 16 h after infection and were then maintained in culture medium. p.i., postinfection.

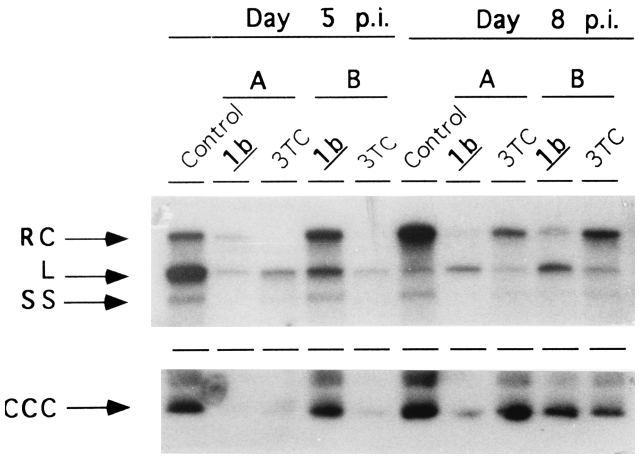

FIG. 3. Southern blot analysis of intracellular DHBV DNA in cultured hepatocytes 5 and 8 days after in vitro infection. Compounds 1b (0.5 μM) and 3TC (0.2 μM) were added 1 h before (A) or 16 h after (B) DHBV infection. Drug treatment was continued for 5 days postinfection. Culture was then maintained without inhibitors for 3 days. Protein-bound and free episomal DHBV DNA was extracted at day 5 and day 8 postinfection (p.i.). RC, relaxed circular DHBV DNA; L, linear DHBV DNA (3 kb); SS, single-stranded DHBV DNA; CCC, covalently closed circular DNA.

infected adjacent hepatocytes, such compounds would have no effect. If liver progenitor cells are uninfected, this kind of compound may prevent the de novo infection of regenerating liver (9). In addition, tetraazamacrocycles can be easily combined with nucleoside analogs such as 3TC (lamivudine), a promising anti-HBV agent (6, 14). In combination, the two classes of antihepadnaviral agents may act additively or synergistically and be able to prevent or delay the emergence of resistant strains.

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