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Inhibitory Effects of Atropine, Protamine, and Their Combination on Hepatitis A Virus Replication in PLC/PRF/5 Cells

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Atropine, protamine, and the combination of these drugs were tested for their effects on hepatitis A virus (HAV) replication in cell culture. PLC/PRF/5 hepatoma cells were treated simultaneously with nontoxic concentrations of these drugs and inoculated with HAV strain CF 53 at several multiplicities of infection. The yields of infectious HAV after 4 and 15 days were markedly reduced by each drug, especially at the lowest multiplicity of infection. The activities of each drug were irreversible. Atropine was active when it was added as late as 2 h after inoculation with HAV. An anti-HAV effect was also induced by treating cells with atropine prior to inoculation. Protamine was active as late as 6 h postinoculation. The combination of atropine and protamine resulted in an enhanced anti-HAV effect. We concluded that these drugs affect undetermined, but separate, steps in the HAV replication cycle.

Infection with hepatitis A virus (HAV) remains a major public health problem both in countries where the disease is sporadic and in those where it is endemic. In industrialized nations such as the United States, HAV infection accounts for up to 50% of all clinical cases of hepatitis (22). In the past few decades, the changing epidemiology of HAV infection in many Western countries (17) has resulted in a lack of naturally acquired immunity to hepatitis A in many adults, creating larger numbers of unprotected individuals. In such populations, epidemic outbreaks with several icteric cases can occur if the virus is reintroduced (34). Although HAV infection during childhood tends to be mild or subclinical, the same infection in adults can be severe and of several weeks' duration (35).

A few studies have been carried out on substances that are active against HAV (14, 28, 29, 31, 35), but no promising antiviral candidates for the treatment of HAV infections have emerged. A study from our laboratory (24) has shown that among 16 substances tested, 7 were shown to be effective against HAV replication in PLC/PRF/5 cells. In another study (7), we have observed that protamine and atropine cause concentration-dependent reductions in the infectivity of HAV. These encouraging results led us to evaluate these two compounds in more detail to determine whether they act additively or synergistically and to determine the step(s) at which they inhibit HAV replication. The results of those studies are presented here.

MATERIALS AND METHODS

Cell culture. Human hepatoma cell line PLC/PRF/5 was grown at 37°C in a 5% CO_2 atmosphere in RPMI 1640 medium (Eurobio, Paris, France) supplemented with 10% heat-inactivated fetal bovine serum (Boehringer GmbH, Mannheim, Federal Republic of Germany) and containing 100 IU of penicillin and 100 μg of streptomycin per ml. After infection, cells were incubated at 32°C in a 5% CO_2 atmosphere, and the maintenance medium contained 1% fetal bovine serum.

Virus. HAV strain CF 53 was isolated in our laboratory from the stools of a patient with HAV infection 3 days after the onset of jaundice (10). This virus was adapted to human hepatoma cell line PLC/PRF/5 by serial passages (11, 12). A virus pool was prepared by infecting confluent layers of PLC/PRF/5 cells with the virus, which was passaged 21 times in this cell line. After 14 days of incubation at 32°C, infected cells and supernatants were submitted to four freeze-thaw cycles, and disruption of cells was achieved by sonication. The virus suspension was clarified by centrifugation at 1,200 × *g* for 10 min at 4°C. This virus pool contained $10^{7.0}$ tissue culture infective doses ($TCID_{50}$s) per ml. The virus stock was stored at −70°C until use.

Antiviral compounds. Atropine [endo(±)-α-(hydroxymethyl)benzeneacetic acid, 8-methyl-8-azabicyclo[3.2.1]oct-yl ester] was purchased from Prolabo (Paris, France), and protamine (grade IV, free base; from salmon sperm) was purchased from Sigma Chemical Co. (St. Louis, Mo.). Before use, the compounds were dissolved in RPMI 1640 medium and sterilized by filtration through a membrane filter (pore size; 0.22 μm; Millex GV; Millipore Corp., Bedford, Mass.) in order to obtain atropine and protamine concentrations of 5.0 and 10.0 mg/ml, respectively. Then, suitable dilutions were made in the same medium.

HAV titration. Fourfold dilutions of each sample were prepared in RPMI 1640 medium; and PLC/PRF/5 cells, which were at confluence in 48-well tissue culture plates (Costar, Cambridge, Mass.), were infected (six wells per dilution) with 100 μl per well. Six wells that received 100 μl of RPMI 1640 medium were used as negative controls. After 1 h of adsorption at 32°C, 1 ml of maintenance medium was added to each well and the culture plates were incubated at 32°C for 49 days with weekly feeding. After 49 days of incubation, cells and supernatants were frozen and thawed four times, and the detection of viral antigen was performed by a solid-phase radioimmunoassay as described previously (9). The infectious HAV titer ($TCID_{50}$ per milliliter) was calculated by the method of Reed and Muench (25).

For experiments with low virus levels ($\leq 10^{2.1}$ $TCID_{50}$s/ml), the infectious HAV titer was evaluated by the same method, but which was modified slightly. The test sample was inoculated into 42 wells containing PLC/PRF/5 cells, and 6 noninfected wells were used as negative controls. The infectious virus titer was estimated by the single-dilution

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method (8), as follows: infectious virus titer = ln {1/[1 − (α/β)]}× 1.443, where α is the number of positive wells, β is the number of wells infected, and 1.443 is the corresponding factor between PFU and $TCID_{50}$. The limit of sensitivity for the single-dilution method was 1.5 $TCID_{50}$/ml.

Drug cytotoxicity evaluations. PLC/PRF/5 cells were grown at 37°C in 24-well tissue culture plates (Costar) to confluency. Six wells were used for each drug concentration, along with an equal number of control (non-drug-treated cells) wells. After washing, the growth medium was replaced with maintenance medium containing the test compound at the appropriate concentration. Cells were exposed to the drug for appropriate times at 32°C (which is the optimal temperature for HAV replication in the PLC/PRF/5 cell line), and each well with maintenance medium containing the drug was renewed every 2 days, to guarantee the presence of a constant concentration of the compounds. At the end of each experiment, cell protein biosynthesis measured by [^{3}H]leucine incorporation (7), the total quantity of cell protein (7), and cellular viability (7) were measured. Moreover, the effects of the drugs on cell growth were also estimated for 4 days at 37°C. The concentration of each compound singly or in combination that reduced these measures by 50% (CD_{50}) was calculated.

Experimental approach for evaluation of drug activity against HAV. Antiviral activity was evaluated, after determination of the infectious virus titer, by calculating the reduction in virus yield. HAV multiplication periods of 4 and 15 days were chosen, according to the growth curve of HAV strain CF 53 in PLC/PRF/5 cells that were not treated with the drugs as a function of the multiplicity of infection (MOI) (unpublished data). At an MOI of 10, the production of infectious HAV increased until day 4 postinfection, and then the virus titer remained constant. At an MOI of 1 the production of infectious HAV increased until day 15 postinfection. At day 4 postinfection, intracellular infectious HAV was present at a sufficient concentration to estimate a drug effect, and infectious HAV appeared for the first time in the cell culture supernatant after the postinoculum washings (data not shown). At an MOI of 1, 4 days is probably close to the time of a single round of HAV strain CF 53 multiplication. At the lowest MOI that was used, infectious cell-associated HAV achieved a sufficient concentration for drug evaluation at day 15 and after multiple rounds of multiplication.

Effect of MOI and HAV multiplication time on antiviral activity. Triplicate wells of 24-well tissue culture plates containing cells at confluency (450,000 ± 40,000 cells per well) were used for each drug concentration and HAV multiplication time. Before infection, cells were washed with RPMI 1640 medium containing the drugs singly and in combination at the maximum nontoxic doses. Then, PLC/PRF/5 cells were infected at the appropriate MOI, and the infection was performed in the presence of the test compounds. After 1 h of adsorption at 32°C and the removal of the excess or unadsorbed virus, 1 ml of maintenance medium containing the appropriate concentration of each drug was added. Then, the culture plates were incubated at 32°C for 4 (MOIs of 10, 1, and 0.1) or 15 (MOIs of 0.1, 0.01, and 0.001) days, and each medium containing the test compound was renewed every 2 days. For each compound, MOI, and HAV multiplication time, triplicate wells were used as controls (virus-infected, non-drug-treated cells). On day 4 or 15, cells were washed twice with RPMI 1640 medium, the virus was extracted by four freeze-thaw cycles, and the HAV suspensions were assayed for infectious virus titer.

The effectiveness of the drug combination against HAV was evaluated by calculating (i) the enhancement of the antiviral activity compared with that of either drug alone, and (ii) the synergistic interaction, which was defined as a decrease in infectious virus yield that was greater than the algebraic sum of the decreases observed with single drugs (19, 21).

Effect of time of drug addition on HAV infectivity. The in vitro time dependency of the antiviral activities of the drugs was studied at an MOI of 1 for 4 days by performing three types of experiments: (i) cell pretreatment with drug which was removed just before infection, (ii) drug addition at different times postinfection and cell incubation in the presence of the drug until the cultures were assayed, and (iii) drug addition at the time of infection and with drug removal at different times postinfection.

Statistical analysis. Statistical analysis of the data was done by using Student's *t* test.

RESULTS

Cytotoxicity studies. For the 4-day exposures of PLC/PRF/5 cells to drugs, the CD_{50}s were about 200 and >1,000 μg/ml and the maximum nontoxic doses were 100 and 500 μg/ml for protamine and atropine, respectively. The combination of the two drugs (100 μg of protamine and 500 μg of atropine per ml) also showed no significant cytotoxic effect ($P > 0.05$) for any of the measures used. For the 15-day cell exposure experiments, the CD_{50}s were about 100 μg/ml for each drug, and the maximum nontoxic doses were determined to be 50 μg/ml for both protamine and atropine. Their combination (50 μg of protamine and 50 μg of atropine per ml) had no significant cytotoxic effect ($P > 0.05$) for any of the measures used.

Antiviral activity of the compounds as a function of MOI. As the MOI was increased, the antiviral effects of the compounds singly and in combination decreased. In all cases and for each MOI studied, the antiviral effect of the combination was greater than the effect of each compound alone. Calculations of the enhancement of antiviral activity and the resulting interactions were performed to determine the effectiveness against HAV attributed specifically to the combination of protamine and atropine. The data for these investigations are summarized in Table 1.

Four days after virus inoculation and only for the lowest MOI tested (0.1 $TCID_{50}$ per cell), the combination of 100 μg of protamine and 500 μg of atropine per ml resulted in an additional reduction in virus yield of 0.15 $\log_{10}$ $TCID_{50}$/ml (positive interaction). For the two other MOIs tested (1 and 10 $TCID_{50}$ per cell) this interaction was slightly negative. However, at the three MOIs tested, the combination showed a significantly enhanced effect ($P < 0.01$) against HAV compared with the effect of either protamine or atropine alone. Thus, the drug combination can be considered synergistic at the lowest MOI tested and additive at the two other MOIs tested.

Fifteen days after virus inoculation and for the three MOIs tested (0.1, 0.01, and 0.001 $TCID_{50}$ per cell), the combination of 50 μg of protamine and 50 μg of atropine per ml acted in a synergistic manner (positive interaction), as shown by the additional reduction in virus yield compared with that from the sum of the effects observed with each drug alone. In addition, when the MOI was increased, the additional decrement caused by the combination decreased. In all cases, enhancement of antiviral activity of the drug combination was significantly different ($P < 0.001$) compared with the effect of either protamine or atropine alone.

TABLE 1. Effect of inoculum size on inhibition of HAV by protamine, atropine, and their combination after a multiplication time of 4 or 15 days at 32°C

Time (days)	MOI	Infectious HAV titer (10^2 $TCID_{50}$s/ml)[a]				Effect of combination (log_{10} $TCID_{50}$s/ml)		
		Control[b]	Protamine[c]	Atropine[c]	Combination[c]	Enhancement of activity compared with that of[d]:		Synergistic interaction[e]
						Protamine	Atropine	
4	0.1	408 ± 68	7.4 ± 1.6 (1.74)	43 ± 8 (0.98)	0.55 ± 0.08 (2.87)	1.13	1.89	0.15
	1	4,079 ± 575	110 ± 18 (1.57)	616 ± 121 (0.82)	18 ± 4 (2.36)	0.79	1.54	−0.03
	10	12,383 ± 1,576	2,008 ± 277 (0.79)	2,818 ± 373 (0.64)	665 ± 109 (1.27)	0.48	0.63	−0.06
15	0.001	937 ± 88	1.3 ± 0.1 (2.86)	174 ± 27 (0.73)	0.1 ± 0.03 (3.97)	1.11	3.24	0.38
	0.01	9,782 ± 468	89 ± 6 (2.04)	2,187 ± 340 (0.65)	10 ± 2 (2.99)	0.95	2.34	0.30
	0.1	30,200 ± 2,275	1,821 ± 206 (1.22)	12,883 ± 1,721 (0.37)	447 ± 53 (1.83)	0.61	1.46	0.24

[a] Values are means ± standard deviations of triplicate experiments. Values in parentheses represent the averages of virus titer decreases (in log_{10} $TCID_{50}$s/ml).
[b] Virus-infected, non-drug-treated cells.
[c] Drug concentrations for the 4-day experiments were as follows: 100 μg of protamine per ml, 500 μg of atropine per ml, and 100 μg of protamine and 500 μg of atropine per ml (combination); drug concentrations for the 15-day experiments were as follows: 50 μg of protamine per ml, 50 μg of atropine per ml, and 50 μg of protamine and 50 μg of atropine per ml (combination).
[d] Values are the additional decrement in virus yield with the drug combination compared with the effect of either drug alone. The enhancement of the antiviral activity was calculated when the decrease in the infectious titers measured for the combination and each drug singly was significantly different ($P < 0.05$).
[e] Additional decrement in virus yield with the drug combination beyond the algebraic sum of the decreases observed with either drug alone.

Induction of an antiviral state by cell pretreatment. To determine a possible prophylactic activity of the compounds against HAV, PLC/PRF/5 cells were pretreated for 24 h before infection with 100 μg of protamine or 500 μg of atropine per ml. Then, cells were infected with HAV, and after 4 days of incubation in drug-free maintenance medium, antiviral activity was evaluated by determination of the infectious virus titer. The results of these experiments are presented in Table 2. In atropine-pretreated cells, the infectious virus production was decreased by 1.22 log_{10} $TCID_{50}$s/ml. In comparison, when 500 μg of atropine per ml was added at the time of HAV inoculation and maintained in cell culture until the end of the 4-day experiments, the infectious titer decrease was 0.82 log_{10} $TCID_{50}$/ml. In contrast, pretreatment of the cells with 100 μg of protamine per ml did not induce an antiviral state, and the slight decrease in infectious virus titer that was observed was not significantly different ($P > 0.05$) compared with that of the control.

TABLE 2. Effect of time of drug addition on the HAV infectivity[a]

Time (±h) of drug addition[b]	Protamine (100 μg/ml)		Atropine (500 μg/ml)	
	Virus yield (10^2 $TCID_{50}$s/ml)[c]	Reduction (log_{10} $TCID_{50}$s/ml)	Virus yield (10^2 $TCID_{50}$s/ml)[c]	Reduction (log_{10} $TCID_{50}$s/ml)
Pretreatment (−24–0)[d]	3,632 ± 380	0.03	244 ± 37	1.22
Simultaneous (+0–96)	104 ± 23	1.57	614 ± 92	0.82
Postinfection				
+0.5–96	93 ± 24	1.62	829 ± 76	0.69
+2–96	124 ± 19	1.49	910 ± 125	0.65
+4–96	120 ± 18	1.51	3,737 ± 631	0.04
+6–96	139 ± 38	1.45	3,956 ± 750	0.01
+8–96	3,159 ± 835	0.09	3,578 ± 569	0.06
+24–96	3,678 ± 783	0.02	3,529 ± 460	0.06
+48–96	3,558 ± 359	0.04	3,655 ± 496	0.05
Reversal[e]				
0–24	352 ± 99	1.04	1,654 ± 468	0.39
0–48	124 ± 15	1.49	1,443 ± 638	0.45
0–72	128 ± 27	1.48	1,373 ± 550	0.47
Control[f]	3,876 ± 587		4,066 ± 612	

[a] PLC/PRF/5 cells were infected at an MOI of 1. Titers of infectious virus in cell samples were determined 4 days after viral inoculation.
[b] −, Drug addition before viral infection; +, drug addition after viral infection.
[c] Values are means ± standard deviations of triplicate experiments.
[d] Cells were pretreated with the indicated concentration of each drug for 24 h at 32°C. Washed cells were then infected and incubated at 32°C with drug-free medium.
[e] Compounds were added at the beginning of infection and removed after the indicated times (in hours) postinfection by washing the cells seven times with RPMI 1640 medium. The drug-free cells then were incubated at 32°C for a total period of 4 days (including the time of drug removal).
[f] Virus-infected, non-drug-treated cells.

Drug addition at different times postinfection. The effect of time of addition of 100 μg of protamine or 500 μg of atropine per ml on HAV replication is also shown in Table 2. Protamine was equally effective when it was added from the time of HAV inoculation to 6 h postinfection. There was no significant difference ($P > 0.05$) between the titer obtained at the time of infection and those obtained with protamine addition at 2, 4, and 6 h postinfection. However, protamine was ineffective when it was added from 8 to 48 h postinfection. No significant difference ($P > 0.05$) was observed between the values obtained in the control and protamine assays. The mean values of the reductions, which were obtained after the addition of 100 μg of protamine per ml until 6 h postinfection, ranged from 1.45 to 1.62 $\log_{10}$ $TCID_{50}$s/ml. Atropine at 500 μg/ml was only effective against HAV replication until 2 h postinfection, and no significant difference ($P > 0.05$) was observed between the measures of the titers obtained from the time of infection to 2 h postinfection after atropine addition, although the decrease seemed to be related to the time of atropine addition. When atropine was added from 4 to 48 h postinfection, no significant effect ($P > 0.05$) was observed on the infectivity of HAV compared with that of controls. The mean reductions in values that were obtained after the addition of 500 μg of atropine per ml until 2 h postinfection ranged from 0.65 to 0.82 $\log_{10}$ $TCID_{50}$/ml. It is noteworthy that a very strong difference in effectiveness was observed between 2 and 4 h postinfection for atropine and between 6 and 8 h postinfection for protamine.

Reversibility of antiviral activity. The reversibility of the antiviral activities of the two compounds was studied by removing the drugs, which were added at the time of HAV inoculation, at various times postinfection (Table 2). The removal of 100 μg of protamine per ml at 48 and 72 h postinfection did not cause a significant loss ($P > 0.05$) of its antiviral activity compared with that obtained by drug removal at the 96 h postinfection (total duration of HAV multiplication). When protamine was removed 24 h postinfection, a slight loss of antiviral activity was observed. The removal of 500 μg of atropine per ml at 24, 48, or 72 h postinfection caused a moderate loss of its activity against HAV compared with that at 96 h postinfection. This loss seemed to be related to the time of atropine removal, although statistical analysis of the data showed no significant difference ($P > 0.05$) in virus yield between the experiments in which drug was removed after 96 h of incubation and those in which drug was removed after 72 or 48 h of incubation (because of the relatively large standard deviations obtained in these experiments).

DISCUSSION

Protamine is a polycationic peptide that is commonly used after several surgical procedures because of its capacity to reverse the anticoagulant activity of heparin (20). Atropine is an anticholinergic agent which is sometimes used when a muscarinic antagonist effect is needed (15). These two compounds are also known to have properties against some viruses (1, 18, 32, 36).

In our experiments, a time dependence of the cells to drug tolerance was observed. The tolerance of the cells to the drugs generally depended on the time of treatment, indicating an additive nature of toxicity (30). When the duration of PLC/PRF/5 cell treatment was extended from 4 to 15 days, the protamine and atropine doses were decreased by 2- and 10-fold, respectively. However, the protamine-atropine combinations did not result in a cytotoxic effect greater than that of either drug alone when used at the same concentrations as those used in the combination. When the two compounds were added at the time of HAV inoculation, they caused inoculum size-dependent reductions in HAV infectivity, regardless of the time of virus multiplication. It is relatively well established that antiviral agents used in combination should possess one or more of the following advantages to be considered therapeutically useful. (i) The combination should not increase or, better yet, should minimize the toxicity relative to that of each of the agents used alone; (ii) they should interact to produce at least an additive or, better yet, a synergistic effect; and (iii) the combination should prevent the appearance of mutants that are resistant to either drug. The combination of atropine and protamine did not increase the toxicity relative to that of each of these drugs used alone. Quantitative evidence for specific antiviral synergism was obtained at the lowest MOIs studied. The greatest synergistic interaction was observed in the 15-day experiments. Moreover, despite progressively higher MOIs, the combination continued to demonstrate an antiviral effect greater than that observed with each of the drugs used alone. When a two-drug combination produces a synergistic effect, it is generally accepted that the two agents have different sites or mechanisms, or both, of biochemical action (2, 6). Conversely, an additive interaction between two drugs may indicate that they share the same mode of action (5, 27). If this commonly held interpretation is correct, then the antiviral effect observed with the protamine-atropine combination suggests that the two agents differ at least in certain aspects of their modes of antiviral action.

We showed that pretreatment of cells with atropine induced an antiviral state unlike that induced by protamine. In addition, this effect against HAV was greater than that obtained when atropine was added at the time of HAV inoculation. Atropine, which is generally known to exhibit pronounced local anesthetic effects by inhibiting cholinergic mechanisms, can induce several effects on cellular membranes, such as inhibition of K^+ efflux and membrane depolarization (16), Ca^{2+} ion displacement (26), an increase in the fluidity of acidic phospholipids (23), and prevention of the increase in Na^+ permeability (13). Such mechanisms, in association with the anticholinoreceptor properties of atropine, might prevent HAV adsorption, penetration, or both to the cell surface and indicate that the cholinergic mechanisms could have a role on these early virus multiplication steps. A possible mechanism that could account for the results obtained with the pretreatment use of atropine would be an alteration of the cell membrane that prevents virus adsorption on cell surface and, subsequently, that inhibits virus penetration into the cell. Another possibility is a competition with infectious HAV for the virus-specific receptor sites. In our study, we showed that atropine addition from 0 to 2 h postinfection resulted in significant antiviral activity. Atropine could exert its antiviral effect not only via inhibition of attachment but may also have interfered with an additional step of the replication cycle if HAV strain CF 53 attachment and penetration in PLC/PRF/5 cells were events as rapid and efficient as those measured by Anderson et al. (3) in BS-C-1 cells and by Wheeler et al. (33) in FRhK-4 cells. These authors have shown that the attachment and penetration of HAV last about 20 min, but after penetration the virion is uncoated very slowly because of a high affinity of the capsid proteins for viral RNA. Since atropine was effective until 2 h postinfection, it is possible that it also acts on HAV uncoating, but additional studies are needed and are in progress in

our laboratory to determine the HAV strain CF 53 adsorption and growth characteristics in PLC/PRF/5 cells, the precise early step(s) of virus replication, as well as the molecular processes that are blocked by atropine.

Protamine, which was added in this study from the time of HAV inoculation to 6 h postinfection, was still able to inhibit infectious HAV yield by more than 1.4 $\log_{10}$ $TCID_{50}$s/ml. It has been reported (32) that protamine is a potent inhibitor of the virion-associated RNA polymerase activity of vesicular stomatitis virus and inhibits endogenous protein kinase activity. Talib and Banerjee (32) suggested that protamine binds at the initiation site of the RNA polymerase and that its primary target is probably the transcriptase but not the replicase. Its site of action on HAV has not yet been determined. It is possible that protamine acts on HAV in a manner similar to that in which it acts on vesicular stomatitis virus, but HAV biochemistry is not understood sufficiently to confirm this hypothesis. In any case, it is evident that the effectiveness of protamine strongly decreased when this drug was added from 6 to 8 h postinfection. Thus, it is reasonable to consider that an important event(s) could happen at this time during the HAV replicative cycle, such as the beginning of accumulation of total viral RNA (3) or the transcription of positive-strand RNA into the negative strands that are used as templates for positive-strand RNA neosynthesis (4).

The reversibility of the antiviral actions of the two compounds was also studied. The results that we obtained indicate that protamine or atropine removal at various times postinfection does not cause a significant loss of their antiviral activities. If the previous hypotheses on the target steps of the two compounds are correct, it is possible that the irreversible antiviral action of protamine may be due to its binding at the initiation site of the virion-associated RNA polymerase, and that of atropine may be due to its fixation on cellular receptors that alter the membrane functions necessary for further virus multiplication steps. A better knowledge of the transcriptional events and the use of radiolabeled atropine should permit verification of these hypotheses.

In summary, our results indicate that protamine and atropine act at the early steps of HAV replication and differ at least in certain aspects of their modes of antiviral action. Atropine has a prophylactic activity probably because it impedes HAV attachment to the cell membrane, but it could also interfere with an additional step of the HAV replicative cycle, that is, HAV uncoating. Protamine might act on the transcription step but would not alter the functions of cell membrane receptors related to virion attachment. Further studies are in progress in our laboratory to determine the precise steps and mechanisms of biochemical action of these two compounds against HAV.

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