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In vitro and *in vivo* activities of T-705 and oseltamivir against influenza virus

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T-705 (6-fluoro-3-hydroxy-2-pyrazinecarboxamide) has a potent and selective inhibitory activity against influenza virus. We studied the effects of an infectious dose on the anti-influenza virus activities of T-705 and oseltamivir, a commercially available neuraminidase inhibitor, both *in vitro* and *in vivo*. Plaque formation of influenza A/PR/8/34 virus was completely inhibited by 10 μg/ml of T-705 after 72 h incubation, whereas visible plaque formation was detected in the plate treated with GS 4071, the active form of oseltamivir (10 μg/ml). The antiviral activity of T-705 was not influenced by an increase in multiplicity of infection (MOI) from 0.0001 to 1, but that of GS 4071 was influenced in a yield reduction assay. No increase in viral yield was seen in either culture supernatant or cells after removal of T-705 (10 μg/ml) but, in contrast, productive infection recurred in culture supernatant and in cells after removal of GS 4071. In mice infected with a high challenge dose of influenza A/PR/8/34 virus, orally administered T-705 (200 and 400 mg/kg/day) completely prevented the death of mice and the survival rates of mice were significantly higher than those in mice treated with oseltamivir ($P<0.01$). When the treatment was delayed at 1, 13 and 25 h post infection, oral administration of 200 mg/kg of T-705 significantly prevented the death of mice ($P<0.01$), and the survival rates of mice treated with T-705 were comparable to those of mice treated with oseltamivir. These results suggest that T-705 has the potential to be a potent inhibitor of human influenza virus infections.

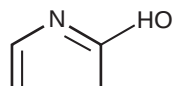
Keywords: influenza virus, T-705, GS 4071, oseltamivir, antiviral activity

Introduction

Influenza is a respiratory infection associated with significant morbidity in the general population and mortality inelderly and high-risk patients (Nichol *et al*., 1999; Thompson *et al*., 2003). Vaccines against influenza virus have been used for prevention of the infection for many years, while amantadine and rimantadine, which inhibit the uncoating process of influenza virus growth (Wang *et al*., 1993), have been available for treatment of the virus. However, these two drugs are not effective against influenza B virus, which does not have an M2 ion channel, and moreover development of drug-resistant viruses occurs rapidly (Hayden *et al*., 1989; Hayden *et al*., 1991). Two neuraminidase inhibitors, zanamivir and oseltamivir, have been approved for use in treatment and prevention of influenza virus infection in adult and children in various parts of the world. Although evidence pertaining to the efficacy of neuraminidase inhibitors in humans is still being accumulated (Ison *et al*., 2001; Pitts, 2002), administration of these drugs should be initiated within 48 h of the first appearance of symptoms in order to be clinically efficacious. In treating with oseltamivir, a correlation between time to intervention and the predicted illness duration was demonstrated in the immediate possibility to access oseltamivir (IMPACT) study. Influenza variants resistant to neuraminidase inhibitors due to mutations within their neuraminidase and haemagglutinin genes have been selected *in vitro* (Blick *et al*., 1995; Gubareva *et al*., 2000; Tai *et al*., 1998) and there is a common concern about the development of drug-resistant virus strains. There has been no serious problem regarding the susceptibility of the drugs to date, but international surveillance is ongoing as neuraminidase inhibitors have a potential for worldwide use (Zambon M *et al*., 2001). If a more reliable method of treating influenza infections is to be realized, a potent anti-influenza virus agent that can completely inhibit virus replication is needed.

T-705 (Figure 1) has a simple structure and displays potent activity against all types of influenza (A, B and C viruses), and excellent therapeutic efficacy in a mice lethal influenza A virus infection model, as we reported previously

Figure 1. Structure of T-705



(Furuta *et al*., 2002). In this study, we investigated the effects of viral multiplicity and removal of compounds on anti-influenza virus activities of T-705 and GS 4071, an active form of oseltamivir *in vitro*. We also investigated how T-705's *in vivo* efficacy was affected by a challenge dose of the virus and by delayed treatment in comparison with oseltamivir.

Materials and methods

Reagents and compounds. T-705 (6-fluoro-3-hydroxy-2-pyrazinecarboxamide) (Figure 1), GS 4071 [(3*R*, 4*R*, 5*S*)-4-acetamido-5-amino-3-(1-ethylpropoxy)-1-cyclohexene-1-carboxylic acid], the active form of oseltamivir, and oseltamivir were synthesized at Toyama Chemical Company, Ltd. (Toyama, Japan). For *in vitro* tests, these compounds were dissolved in Eagle's modification of minimum essential medium [(EMEM), Sigma Chemical Co., St Louis, Mo., USA] and then further diluted in each *in vitro* test medium. For *in vivo* tests, they were suspended in 0.5% methylcellulose.

Cells. Madin-Darby canine kidney (MDCK) cells were purchased from American Type Culture Collection (ATCC) (Manassas, Va., USA). Cells were routinely passaged in supplemented with 10% fetal calf serum [(FCS), Iwaki, Tokyo, Japan] and 60 µg/ml kanamycin.

Virus. Influenza A/PR/8/34 (H1N1) virus was kindly donated by Dr Y Okuno (Osaka Prefectural Institute of Public Health) and propagated in MDCK cells.

Plaque assay. Plaque assays were performed essentially as previously reported (Woods *et al.*, 1993). MDCK cell monolayers grown in 6-well tissue culture plates were inoculated with 70 plaque forming unit (PFU) of virus, which was corresponding to a multiplicity of infection (MOI) of 0.0001. After adsorption at 35°C for 1 h, the inoculum was removed, and the cell monolayers were overlaid with EMEM containing 0.5% agarose, 0.001% DEAE-dextran, 2 µg/ml TPCK-treated trypsin (Sigma) and 10 µg/ml T-705 or GS 4071. These doses corresponded to about 60 times and about 2500 times as higher as the IC_{50} of T-705 (0.16 µg/ml) and oseltamivir (0.0039 µg/ml) against A/PR/8/34 virus replication, respectively. The plates were incubated at 35°C in 5% CO_2, for 48 h and 72 h, and were fixed with 3% formaldehyde and stained with 0.005% amido-black. Plaque size was visually inspected and photographed.

Effect of MOI on antiviral activity. Inhibition of viral growth was assessed by the yield assay. The monolayers of MDCK cells in 24-well tissue culture plate were inoculated in duplicate with MOI of 0.0001 (14 PFU/well), 0.01 (1400 PFU/well) or 1 (140 000 PFU/well). After 60 min, the inoculum was removed and the test medium containing 1 and 10 µg/ml of T-705 or GS 4071 was added. The test plates were incubated under 100% humidity and 5% CO_2 in an overlay medium, containing 1% BSA and 2 µg/ml trypsin, at 35°C for 10, 20 and 30 h. At each time, the test plates were frozen at –80°C. After two freeze-thaw cycles, the viral yield of these test plates was determined by the plaque assay. In cases in which viral yield was less than the limit of detection (2.5 PFU/well), the yield was approximated to 2.5 PFU/well.

Virus regrowth after removal of the compounds. The monolayer of MDCK cells in 24-well tissue culture plates were inoculated in duplicate with a MOI of 0.001 (140 PFU/well) of influenza A/PR/8/34 virus. After 60 min, the inoculum was removed, and the test medium containing 1 and 10 µg/ml of T-705 or GS 4071 was added (Furuta *et al*., 2002). The test plates were incubated under 100% humidity and 5% CO_2 in an overlay medium containing 1% BSA, and 2 µg/ml trypsin at 35°C for 10 h (0–10 h) and then each compound was rinsed three times with the compound-free medium. The virus yield in the supernatant and that were associated with the cells were determined by plaque assays at 10, 20 and 30 h post infection. In cases in which viral yield was less than the limit of detection (5 PFU/well), the yield was approximated to 5 PFU/well.

Therapeutic efficacy in mice. Male BALB/c mice (weight 17–19 g) were anaesthetized by diethyl ether and exposed to a mouse-adapted influenza A/PR/8/34 virus by intranasal instillation (Furuta *et al*., 2002).

In the experiment with a high-challenge dose, 70 mice were infected with the virus with 3×10^4 PFU (which corresponds to >1000-fold of the LD_{50}) and divided into five groups (n=14). The mice were orally administered with T-705 and oseltamivir at doses of 200 and 400 mg/kg/day (four times a day, 6 h interval) for 5 days beginning 1 h after infection. As controls, mice were treated with 0.5% methylcellulose. The number of surviving mice was observed daily for 21 days after infection.

The effect of delayed treatment on the survival rate of mice was examined. Mice were infected with the virus with 3×10^2 PFU and were divided into seven groups (n=14) on day 0. The mice were orally administered with T-705 and

oseltamivir at a dose of 200 mg/kg/day (four times a day, 6 h interval) beginning 1, 13 and 25 h after infection to day 4. As controls, mice were treated with 0.5% methylcellulose solution beginning 1 h after infection. The number of survival mice was observed daily for 21 days after infection. Differences in survival rates of mice were analysed by log-rank test in both experiments.

We conducted procedures in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals and the experimentation guidelines of the Toyama Medical and Pharmaceutical University.

Results

Comparison of plaque inhibition between T-705 and oseltamivir during prolonged incubation. The sizes of plaque at 48 and 72 h after infection are shown in Figure 2. At 48 h after infection, both T-705 and GS 4071 at a concentration of 10 μg/ml inhibited plaque formation of influenza A/PR/8/34 virus. At 72 h after infection, plaque formation continued to be inhibited by T-705, whereas visible plaque formation was detected in the plate treated with GS 4071.

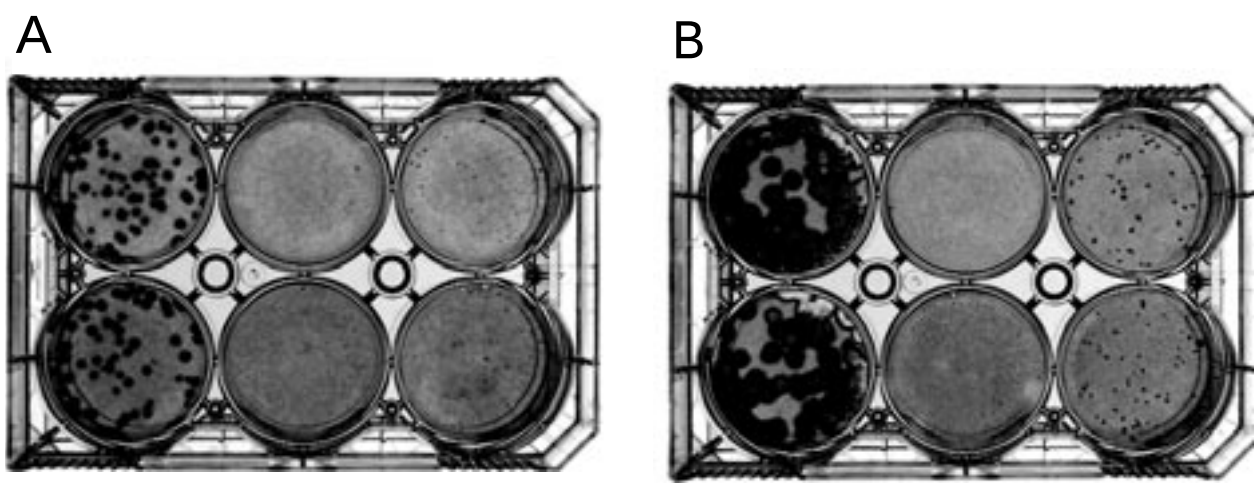
Effect of MOI on antiviral activity. The inhibitory effects of T-705 and GS 4071 on viral growth were evaluated within a wide range of MOI with influenza A/PR/8/34 virus (from 0.0001 to 1) (Figure 3). Viral growth was inhibited by T-705 at a dose of 1 μg/ml at all multiplicities, and the antiviral activity of T-705 was not affected by an increased MOI. Viral growth was incompletely inhibited by GS 4071 even at a low MOI of 0.0001, and the activity of GS 4071 appeared to be influenced by virus multiplicity.

Virus regrowth after removal of the compounds. The viral yields both in supernatant and in cells after removal of compounds were shown in Figure 4. After 10 h incubation with T-705 at doses of 1 and 10 μg/ml, an increase in viral yield was inhibited both in the supernatant and in the cells. In the culture treated with T-705 at a dose of 10 μg/ml for 10 h, no productive infection was observed even 20 h after removal of the compound (30 h post infection). Although sequential increases of yields were seen both in the supernatant and in the cells treated with T-705 at a dose of 1 μg/ml, they were kept about 100 to 1000 times lower than those seen in controls. In contrast, in the culture treated with GS 4071 at a dose of 10 μg/ml, an increase in viral yield was inhibited only in the supernatant after 10 h incubation. After removal of GS 4071, productive infection occurred again both in the cells and in the supernatant.

Therapeutic efficacy of T-705 in mice. In mice infected with a high challenge dose of influenza A/PR/8/34 virus (>1000-fold of LD_{50}), oral treatment of T-705 at doses of 200 and 400 mg/kg/day completely prevented the death of mice (Figure 5). Both doses were significantly superior to those of mice treated with two doses of oseltamivir (7 and 21% survival, respectively).

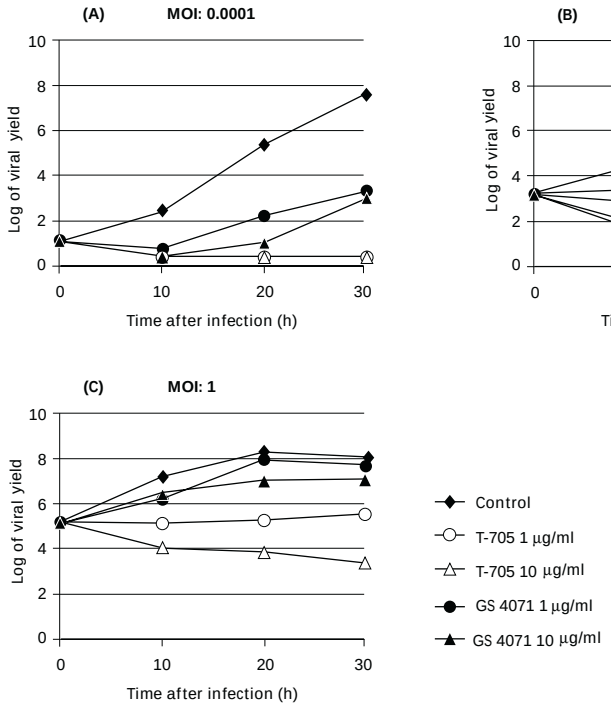
In the delayed treatment model, oral administration of 200 mg/kg/day of T-705 and oseltamivir significantly prevented the death of mice even when the treatment delayed at 25 h post infection (Figure 6). The survival rates of mice treated with T-705 at 1, 13 and 25 h after infection were 100, 100 and 71%, respectively. These values were always higher than the survival rates of mice treated with oseltamivir (93, 79 and 50% survival, respectively), although not statistically significant.

Figure 2. Inhibitory effects of T-705 and GS 4071 on plaque formation of influenza A/PR/8/34 virus



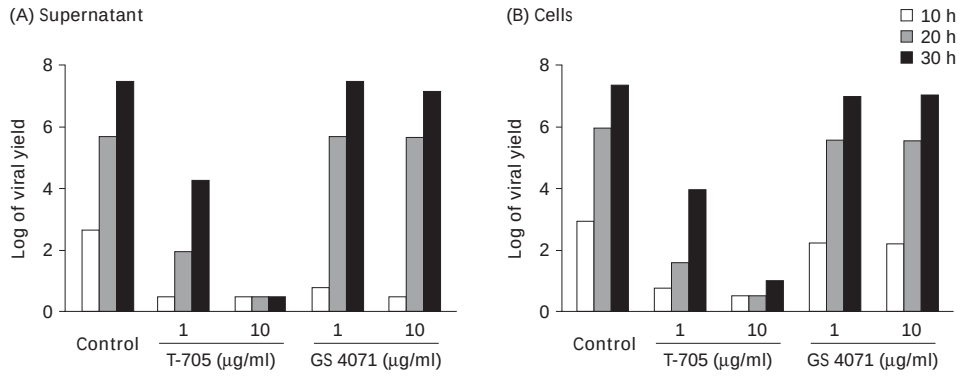
MDCK cells were treated with T-705 and GS 4071 after being infected at 70 PFU per well as described in the text. After 48 (**A**) and 72 (**B**) h of treatment, the plates were fixed and stained. Left wells, control; centre wells, T-705 10 μg/ml; right wells, GS 4071 10 μg/ml.

Figure 3. Effect of virus MOI on anti-influenza A/PR/8/34 virus activities of T-705 and GS 4071



MDCK cells were infected with influenza A/PR/8/34 virus at multiplicities of infection of 0.0001 (**A**), 0.01 (**B**) and 1 (**C**). The infected cultures were treated with T-705 and GS 4071 at doses of 1 and 10 μg/ml. At 10, 20 and 30 h post-infection, the viral yield in the wells was determined. The results presented were obtained from a representative experiment.

Figure 4. Viral yields in supernatant and cells after removal of T-705 and GS 4071



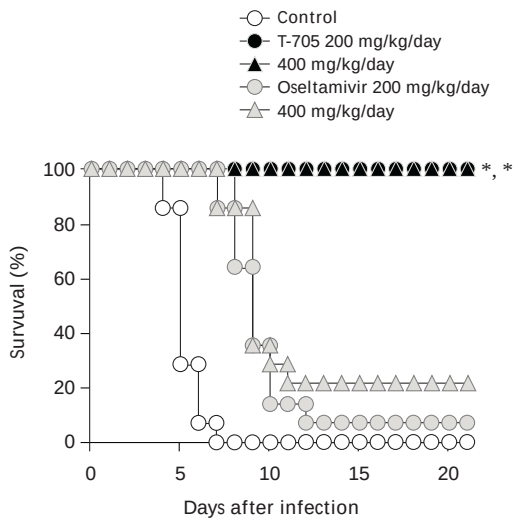
MDCK cells were infected with influenza A/PR/8/34 virus at a multiplicity of infection of 0.001. The cultures were treated with T-705 and GS 4071 at doses of 1 and 10 μg/ml for 10 h (0–10 h) and then the compounds were rinsed. Viral yields in the supernatant (**A**) and the cells (**B**) were determined by plaque assays at 10, 20 and 30 h post-infection.

Discussion

In this report, we investigated the anti-influenza profile of T-705 and oseltamivir (or its active compound, GS 4071), a commercially available neuraminidase inhibitor, in some *in vitro* and *in vivo* studies and demonstrated that T-705 exhibited potent antiviral activity, especially in severe influenza virus infections.

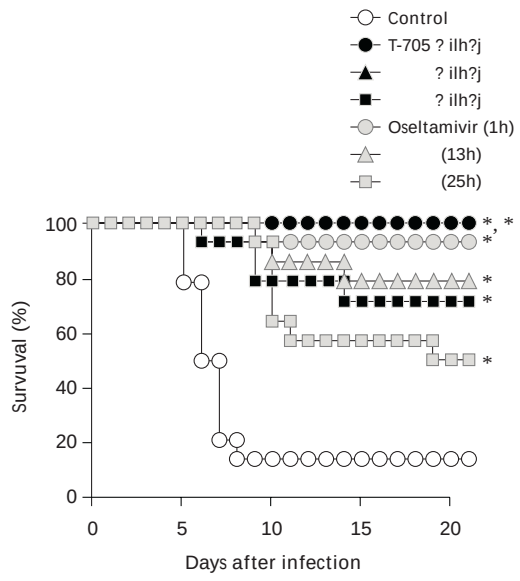
In *in vitro* experiments, 10 μg/ml of oseltamivir did not inhibit virus plaque formation or viral growth at a high multiplicity. Production of progeny virus was observed after removal of the compound. The dose of oseltamivir corresponds to about 2500-fold of the IC_{50} for replication of A/PR/8/34 virus. Similar observations have been reported. Anti-influenza A/Sydney/05/97 (H3N2) virus activities of GS 4071 and other neuraminidase inhibitor RWJ-270201

Figure 5. Therapeutic effects of T-705 and oseltamivir in mice infected with a high challenge dose of influenza A/PR/8/34 virus



Mice were infected with 3×10^4 PFU of influenza A/PR/8/34 virus and were orally administered with T-705 and oseltamivir at doses of 200 and 400 mg/kg/day for 5 days beginning 1 h post-infection (n=14). The results presented were obtained from a representative experiment.
*P<0.01, compared to 0.5% methylcellulose solution-treated controls and oseltamivir-treated groups (log-rank test).

Figure 6. Effect of delayed treatment with T-705 and oseltamivir on an influenza A/PR/8/34 virus infection in mice



Mice were infected with 3×10^2 PFU of influenza A/PR/8/34 virus, and were orally administered with T-705 and oseltamivir at a dose of 200 mg/kg/day from 1,13 and 25 h post-infection to day 4 (n=14). The results were obtained from a representative experiment.
*P<0.01, compared to 0.5% methylcellulose solution-treated controls (log-rank test).

were dependent upon the virus MOI (from 0.00018 to 0.0225), and their activities attenuated >1000-fold at the highest MOI. Moreover, RWJ-270201 inhibited the production of an extracellular virus but not of a cell-associated virus in yield reduction studies using influenza A and B viruses (Smee *et al.*, 2001). Inhibitory activity of zanamivir against influenza A/Virginia/88 (H3N2) virus was assessed in MDCK cells after overnight incubation by a yield reduction assay. In supernatant fluid, virus recovery was completely inhibited by zanamivir at the concentration of 1.0 µg/ml and above, but reduction in cell-associated virus yield was smaller than that in supernatant at all tested concentrations (Hayden *et al.*, 1994). On the other hand, the inhibitory activity of T-705 was not influenced by virus challenge dose and production of progeny virus was not seen after removal of the drug.

Significantly, T-705 prevented the death of mice in our infection models. Virus challenge dose or delay of treatment initiation did not affect the efficacy of T-705, but the efficacy of oseltamivir was affected by virus challenge dose. Sidwell has also shown the effect of virus challenge dose on the efficacy of delayed oseltamivir therapy. At a high challenge dose ($10^{5.6}$ $CCID_{50}$/ml) of influenza A/NWS/33 (H1N1) virus, the efficacy of delayed oseltamivir therapy attenuated as compared to that at a moderate challenge dose ($10^{3.6}$ $CCID_{50}$/ml), which was an approximate LD_{85} in mice (Sidwell *et al.*, 1998). It was also shown that RWJ-270201 did not prevent the death of mice infected with influenza A/Shangdong/09/93 (H3/N2) virus at the highest challenge dose corresponding to approximately twice the LD_{100} (Sidwell *et al.*, 2001). Oral administration of RWJ-270201 and oseltamivir completely prevented the death of mice at 24 h post infection, but no significant prevention of lethality was seen when treatment was started at 48 h post-infection (Bantia *et al.*, 2001). It was reported that virus strain affected the therapeutic efficacy of RWJ-270201 in a mice infection model. The survival rates in mice infected with A/PR/8/34 virus strain appeared to be lower than the rates in mice infected with A/Bayern/07/95 (H1N1) virus strain, in spite of similar *in vitro* inhibitory activities (Sidwell *et al.*, 2001). Using A/PR/8/34 virus might be one of the reasons that treatment dose of oseltamivir in our experiments tended to be higher than earlier reported studies (Sidwell *et al.*, 1998). It has been reported that various cytokines are produced both locally and systemically in the mice infection model (Hennet *et al.*, 1992; Tsurita *et al.*, 2001). These protective immune responses might have been induced during our experiments and influenced virus yield and mice mortality, especially when treatment began late in the infection.

With a different mode of action from neuraminidase inhibitors, ribavirin shows anti-influenza virus activity. Ribavirin inhibited the production of new virions through its 5′-triphosphate and 5′-monophosphate (Eriksson *et al.*,

1977; Streeter *et al*., 1973). In other reports, ribavirin blocked both extracellular and cell-associated virus yields and its efficacy was not affected by increasing the MOI (Smee *et al*., 2001). Browne *et al*. demonstrated that the efficacy of ribavirin at a high-multiplicity influenza virus infection corresponds to MOI of 5 (Browne *et al*., 1983). Neuraminidase inhibitors inhibit the spread of virus by retaining virions on the membrane surface, but do not inhibit the production of progeny virions (Gubareva *et al*., 2000; Palese *et al*., 1976).

In conclusion, T-705 completely inhibited the production of progeny virions and showed potent *in vitro* and *in vivo* anti-influenza virus activity. In our preliminary study, some nucleic acid interfered with antiviral activity of T-705 (data not shown) and we consider that T-705 is likely a nucleotide inhibitor of RNA synthesis. Detailed studies of T-705's inhibition of RNA synthesis and effect on polymerase activity of influenza virus are in progress to determine the mechanism of action. We believe T-705 to be a potent and unique inhibitor against human influenza infections.

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