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Antiviral Activity *in Vitro* of Kutapressin against Human Herpesvirus-6

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Abstract. *The recently discovered human herpesvirus-6 (HHV-6) is being associated with an increasing number of conditions in which there is evidence of immunologic dysfunction. A number of widely available antiviral agents have shown little or no activity against the virus. We found that Kutapressin (KU), a drug that has been available to practicing physicians for over 50 years, has potent, previously unexpected antiviral effects. Cells known to allow replication of HHV-6 were infected with the virus, under various conditions. Either pretreatment of the cells prior to infection or treatment shortly after infection, inhibited viral replication by >90%. Indirect evidence suggests that KU may inhibit viral attachment to cellular receptors, and inhibit intracellular maturation of the virus. Given these in vitro findings, and the low frequency of toxicity reported with the use of KU, clinical trials of this drug in patients with evidence of reactivated HHV-6 infection would seem to be warranted.*

Human herpesvirus-6 (HHV-6) was originally isolated and described in 1986 (1). Many different strains of the virus, comprising at least two families (the A and B variants) with different biologic properties, have been identified. Sero-epidemiological studies have shown that infection with the virus is widespread. Most people become permanently infected with HHV-6 in childhood. Initial primary infection with HHV-6 may be asymptomatic; in infants, it may lead to exanthem subitum (2) or less specific acute febrile illnesses (3); in older children and young adults, it may lead to a mononucleosis-like illness (4). Thereafter, the infection becomes dormant within leucocytes, monocytes/macrophages and possibly various epithelial tissues, producing no apparent morbidity (5). Under certain circumstances, however, HHV-6 infection becomes reactivated: 1) levels of IgG and IgM antibodies to the virus rise; 2) lymphocytes placed into primary cell culture develop a characteristic cytopathic effect (the outgrowth of refractile "giant cells" after 3-5 days in culture); 3) the giant cells stain with monoclonal antibodies to HHV-6 and demonstrate HHV-6 nucleic acids by Southern blotting or polymerase chain reaction; 4) electron microscopy reveals virus budding at the inner nuclear membrane into the cisternae of the rough endoplasmic reticulum, from the cytosol into Golgi vesicles or exiting from the cell membrane; and 5) demonstrates passage of the cytopathic effect when uninfected cells are exposed to cell-free supernatant from primary cell cultures (5). Such evidence of HHV-6 reactivation has been reported in a variety of conditions, all of which are known or suspected to be associated with immunologic dysfunction: the acquired immunodeficiency syndrome (AIDS), Hodgkin's disease, autoimmune disorders (Sjögren's Syndrome, systemic lupus erythematosus), Kikuchi Syndrome, atypical polyclonal lymphoproliferation, post-organ transplantation, and in the chronic fatigue syndrome (CFS) (5, 6). Like human immunodeficiency virus-1 (HIV-1), HHV-6 is tropic for $CD4^+$ T cells; dual infection of $CD4^+$ cells with both HIV-1 and HHV-6 greatly increases the rate of CD4+ cell death (7). Whether the reactivation of HHV-6 in states of immunologic dysfunction, or its synergistic effects with HIV-1, contributes to pathology or morbidity remains to be determined.

Given the potential pathological role of HHV-6, it is important to find pharmaceuticals which inhibit the replication of this virus. Unfortunately, previous studies have shown that acyclovir (ACV), zidovudine (AZT), 5'mercurithio-2-deoxyuridine (HgdUrd), and glutathione-HgdUrd are ineffective against HHV-6 replication (5). Ganciclovir may have some activity against certain strains of HHV-6, but not others (8, 9). Phosphonoacetic acid and phosphonoformic acid are able to inhibit HHV-6 infection (10); however, these pyrophosphate analogues have considerable toxicity.

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Table I. *In vitro inhibition of HHV-6 by kutapressin (KU).*

Treatment*	Concentration of KU (μg/ml)	% of cells positive (average) for HHV-6 antigen by IFA**			% inhibition of HHV-6 infection by day 14
		3 days	7 days	14 days	
1. HSB_2 cells were infected with HHV-6 and carried in medium without KU	none	31	62	>90	—
2. HSB_2 cells were treated with KU overnight, then infected with HHV-6 and then carried in medium without KU	300	6	22	55	38.9
	500	4	15	35	61.1
3. HSB_2 cells were treated with KU overnight, then infected with HHV-6 and then treated continuously with KU	300	0	0	3	96.7
	500	0	0	1	98.9
4. HSB_2 cells were infected for 2 hrs with HHV-6, washed, then treated continuously with KU	300	2	4	8	91.1
	500	1	1	3	96.7
5. HSB_2 cells were infected with HHV-6, then after 3 days treated continuously with KU	300	—	8	70	22.2
	500	—	5	60	33.3
6. HSB_2 cells (1×10^6) were treated continuously with KU, but never infected†	300	0	0	0	N/A
	500	0	0	0	N/A
7. HSB_2 cells were carried in medium without KU	—	0	0	0	N/A

*A1000 $TCID_{50}$/ml of HHV-6 (GS strain) was used to infect the cells. The cells were treated with virus for 2 hours at 37°C. In case of pretreatment with KU, the 1×10^6 cells showing >92% viability were treated overnight, washed and then infected with HHV-6 and carried in KU. KU at 50 μg & 100 μg levels did not inhibit HHV-6 infection effectively (25-30%).

**Infection of HHV-6 was monitored (a) by microscopic examination of cells for formation of large giant cells and (b) by detecting HHV-6 antigen positive cells by IFA, using two monoclonal antibodies (P41/38, gp 116/64/54) and one HHV-6 positive human serum (N901). Results are an average of 3 experiments. Selected pre- and post- KU treated cells were processed for ultrastructural studies.

†No toxicity was noted: cells remained >90% viable after 14 days, as determined by trypan blue staining.

The uncontrolled experience of several clinicians has suggested that a drug called Kutapressin™ (KU) might produce symptomatic improvement in some patients with chronic fatigue syndrome, an illness in which the reactivation of HHV-6 has been reported (6). Because CFS has features suggesting a chronic viral infection, we decided to determine whether KU might have antiviral activity. KU is a prescription drug (11) consisting of processed porcine liver extract, containing peptides. Previous research has indicated that KU may speed the resolution of herpes zoster (11), and one report found that it might have therapeutic value in patients with neurasthenia (12), an illness that is clinically similar to CFS. KU has been used in patients in the United States since 1939 without reports of significant toxicity.

Materials and Methods

Cell line. HSB_2, an immature T-cell line expressing $CD_{3\delta}$ receptors, was infected with HHV-6. This cell line permits active replication of the HHV-6 type A variants. By day 14, >90 of the cells are infected, as detected by rising monoclonal antibodies (5, 13). We did not use human cord blood mononuclear cells because we have found the magnitude of infection after PHA stimulation of these cells to vary considerably from one batch of cord blood to the next.

HHV-6 isolates. A 1000 $TCID_{50}$/ml of the GS strain was initially used. The study was also repeated using a type B variant of HHV-6 (Z-29) and the MOLT-3 cell line, a mature T-cell line expressing CD4+ receptors. The GS strain of HHV-6 does not grow in MOLT-3 cells, and the Z-29 strain does not productively infect HSB_2 cells (5).

Infection of cells with HHV-6. As stated in the Table I legend, >90% viable cells were washed with RPMI-1640 medium containing no FCS. The cells were then pelleted and infected with the virus (1000 $TCID_{50}$/ml) for 2 hours at 37° C. During the virus absorption period the cells were gently shaken 3-4 times. After virus absorption, the cells were pelleted and residual virus was removed.

The presence of active HHV-6 infection in the HSB_2 or MOLT-3 cells was shown by the development after 3-5 days of the characteristic cytopathic effect, formation of giant cells, which usually appear singly,

Table II. *In vitro effect of kutapressin (KU) on HHV-6. HSB_2 were simultaneously infected with the virus and treated with the drug (the average of 2 experiments)**

Dose of KU	Days post infection	Cell viability (%)	Cell count	% antigen positive cells	% inhibition
100 μg	3	81.8	3.6×10^5	5	
	14	73.0	1.7×10^6	48	47.8
300 μg	3	83.4	3.8×10^5	2	
	14	73.8	1.7×10^6	9	90.2
500 μg	3	80.1	2.5×10^5	1	
	14	72.0	1.05×10^5	3	96.7
Virus only	3	78.8	4.5×10^6	8	
	14	60.0	$52.\times10^5$	92	N/A
Drug only					
300 μg	3	92.2	2.93×10^6	None	
	14	81.0	2.2×10^6	None	N/A
500 μg	3	93.2	2.7×10^6	None	
	14	90.5	2.45×10^6	None	N/A

*A 1000 $TCID_{50}$/ml of GS strain of HHV-6 was used. 5×10^6 cells were first pelleted and then KU and virus were added together. The cells were allowed to incubate for 2 hours before cell culture medium was added and incubated at 37°C. The HHV-6 infected cells were followed by Mabs, as in Table 1.

which express HHV-6 antigens, as determined using a polyclonal human serum (N901) at 1:20 dilution (titer 1:320) and two HHV-6 monoclonal antibodies (13) to early (p41) and late (gp 116/64/54) viral proteins. We could not solely use the giant cell assay to evaluate the results because only 60-65% of the infected cells, as determined by antigen expression, become giant cells. The cells were evaluated by indirect immunofluorescence assay (IFA). At least 10 fields were evaluated to obtain an average count of infected cells. All infected and uninfected cell cultures were carried in RPMI-1640 containing 10% Δ fetal calf serum (FCS) and regular amounts of antibiotics. The cell culture medium was changed at least twice during the 14 days of the experiment. To change the cell culturing medium, the cells were centrifuged at low speed (1000 RPM) for 5 minutes, and resuspended in medium with and without KU.

Cell toxicity. In order to evaluate the effect of KU on HSB_2 or MOLT-3 cells, the cells were tested for cell viability using trypan blue stain. The total number of viable cells were counted before and after the initiation of the experiments.

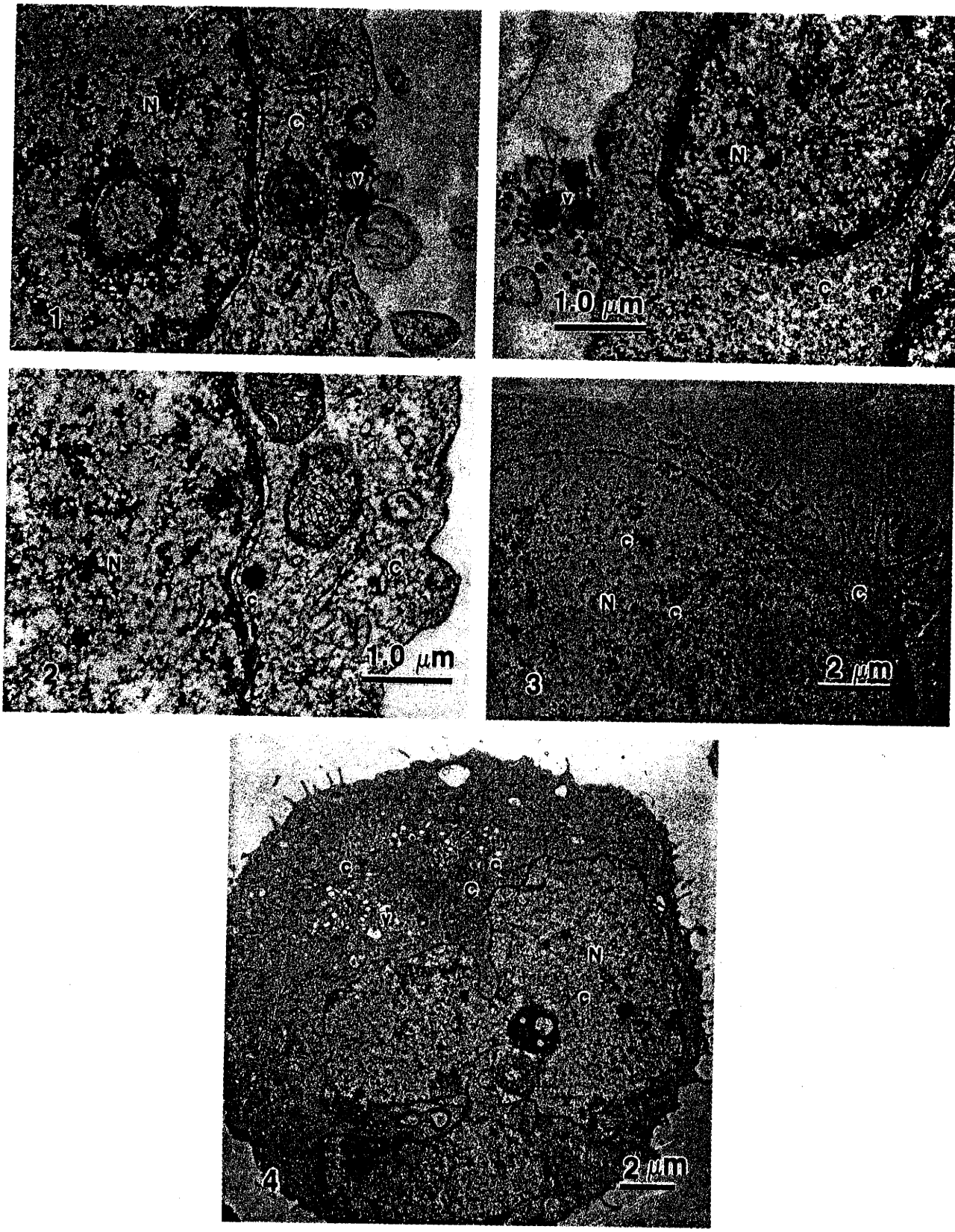
Kutapressin. KU free of phenol (lyophilized) was provided by Schwarz Pharma, Mequon, WI. KU was dissolved in RPMI-1640 medium, with antibiotics and without serum, and was stored at 4°C. Before it was used, the bottle containing KU was shaken thoroughly and then centrifuged at 2000 RPM for 10 minutes, in order to eliminate any precipitate which may have been formed.

Electron microscopy. HHV-6 infected cells (KU treated and untreated) were prepared for transmission electron microscopy according to the procedure of Biberfeld *et al* (14). The cells were briefly fixed in cacodylate buffered glutaraldehyde, post-fixed in Dalton's chrome osmium (15) and stained with aqueous uranyl acetate. The cells were dehydrated in ethanol followed by propylene oxide, infiltrated and embedded in Epon (Tousimis Research Corporation, Rockville, MD). Thin sections were cut on an LKB Ultrotome III ultramicrotome and stained with aqueous uranyl acetate and lead citrate. The specimens were examined and photographed in a Siemens Elmiskop 1A electron microscope.

Results

When the cells were infected with HHV-6 *without* adding KU to the culture medium over 90% of the cells became infected by the 14th day after the initial infection (Table I, Treatment 1). In marked contrast, when the cells were pretreated with KU overnight, then infected and cultured without adding KU to the medium, more than 60% (38.9% inhibition) of the cells became infected by day 14 (Table I, Treatment 2). When the cells were treated with KU both before and then continuously after infection, there was >95% inhibition of infection, with either the 300 μg/ml, or 500 μg/ml concentrations of KU (Table I, Treatment 6). Similarly, when the cells were first infected with HHV-6 for two hours and then treated with KU continuously thereafter, there was >90% inhibition of infection, with either the 300 or 500 μg/ml concentrations of KU (Table I, Treatment 4). When treatment with KU was delayed until three days after infection, however, inhibition of HHV-6 replication was considerably less marked (Table I, Treatment 5). Neither uninfected HSB2 cells treated continuously for two weeks with KU nor uninfected HSB2 cells carried in culture without KU showed any evidence of HHV-6 antigens (Table I, Treatments 6 and 7). When HSB2 cells were treated continuously with KU at these concentrations, there also was no evidence of toxicity. We repeated the same experiments with Z-29, one of the type B variants of the virus (5), with similar results (data not shown). Table I shows as average of three experiments. The variability between experiments was insignificant because the number of infected cells as detected by the monoclonal antibodies were fewer than 1% of the total.

Table II shows that when the cells were simultaneously infected with HHV-6 and treated with KU a significant inhibition of HHV-6 infection was observed with 300 and 500 μg/ml of KU (>90%). Even at 100 μg/ml of KU, >47% inhibition of HHV-6 infection was observed. Table II also shows that



Figures 1- 4

the cells viability at 14 days post-treatment was ≥72%, compared to >81% of uninfected KU treated cells. In the absence of KU the HHV-6 infected cells had a viability of 60%. This suggests that infected cells in the presence of KU are living longer and KU did not allow the release of virus from the infected cells. These data also suggest that KU competed with the virus in perhaps blocking the HHV-6 absorption to the cells.

Since pretreatment of cells with KU before HHV-6 infection showed ≥95% inhibition of infection, suggesting that KU may be blocking cellular receptors for the virus, thereby inhibiting virus attachment and penetration. This observation was supported by electron micrograph, because cells pretreated with KU, prior of infection showed large numbers of extracellular HHV-6 virions, whereas intracellular virions were rarely seen by electron microscopic examination (Figure 1). In contrast, when KU treatment was started following the initial 2-hour infection with HHV-6, fewer extracellular virions were seen. At 500 μg/ml only a few nucleocapsids were seen in the cytoplasm adjacent to the nuclear envelope (Figure 2), possibly indicating a partial inhibition in the uncoating of the viral genome in these cells. At 300 μg/ml some cells contained numerous intranuclear nucleocapsids (Figure 3) but no intracisternal or intracytoplasmic HHV-6 particles, indicating that viral replication was arrested at the intranuclear stage.

When HSB2 cells were first infected with HHV-6, allowed to proceed with infection for 3 days, and then treated with KU, a partial reduction in the number of HHV-6-infected cells was evident, suggesting that KU inhibited further cell-to-cell infection of HHV-6 (figure not shown). Another observation also supported this possibility: when KU was removed from virus-infected cells after 14 days, and then the cell culture was allowed to continue for another 7 days, HHV-6 infection reappeared but progressed slowly (data not shown), compared to the rate at which the number of infected cells grew without KU treatment. Finally, when HHV-6-infected cells were grown in the absence of KU, HHV-6 could be found in all stages of replication (Figure 4).

Figure 1. *HSB_2 cells pretreated with KU prior to infection with HHV-6. Only extracellular virions were seen. (The left panel involves treatment with 300 μg/ml).*
N-Nucleus, C-Cytoplasm, V-enveloped HHV-6 virions.

Figure 2. *HSB_2 cells infected with HHV-6 for two hours, washed, and then treated with 500 μg/ml KU. A few intracellular nucleocapsids are seen in the cytoplasm adjacent to the nuclear envelope.*
N-Nucleus, C-Cytoplasm, c-capsid of HHV-6.

Figure 3. *HSB_2 cells infected with HHV-6 for two hours, washed, and then treated with 300 μg/ml KU. Compared to treatment with the 500 μg/ml concentration numerous intranuclear nucleocapsids are seen.*
N-Nucleus, C-Cytoplasm, c-capsid of HHV-6.

Figure 4. *HSB_2 cells infected with HHV-6; no treatment with KU. Virions in all stages of replication are apparent.*
N-Nucleus, C-Cytoplasm, c-capsid of HHV-6, v-enveloped HHV-6 virions.

Discussion

Our data show that KU, at concentrations of 300 and 500 μg/ml, is a potent *in vitro* inhibitor of HHV-6. The inhibition is more evident when cells are pretreated with KU prior to infection with HHV-6 (>95% inhibition), and KU competed with HHV-6 when KU and HHV-6 were added together to the cells. When the cells were allowed to first proceed with HHV-6 infection, KU was able to prevent cell-to-cell infection by not allowing cells to produce extracellular virus. Although the mechanism of *in vitro* action of KU on HHV-6 is unknown, the electron microscopy data support the fact that it is capable of blocking HHV-6 absorption and penetration, and inhibiting viral maturation when HHV-6 was first adsorbed to the cell.

There is some suggestion that KU may also have antiviral properties against other human herpesviruses. Preliminary data in our laboratory has found that concentrations of 300 and 500 μg/ml of KU block the induction of early antigens to Epstein-Barr virus in Raji cells (an EBV genome-positive, nonproducer B cell line) as well as immortalization of cordblood mononuclear cells by B95-8 strain of EBV. To our knowledge, possible antiviral activity of KU against Herpes simplex virus and cytomegalovirus has not been reported. Clinical studies have reported that KU may speed the resolution of herpes zoster (16), suggesting the possibility of activity against varicell zoster virus.

The implications of these *in vitro* findings for clinical practice are unclear, for several reasons. First of all, the pediatric conditions in which HHV-6 is clearly an etiologic agent are all brief, self-limited illnesses in which even effective antiviral therapy may do little to speed the resolution of symptoms. Only controlled clinical studies of this widely available drug can provide evidence of clinical benefit.

Second, although these data suggest the possibility that KU might have therapeutic value in the several diseases involving immune dysfunction with which HHV-6 has been associated, it is unclear whether the virus directly contributes to the pathologic process. Furthermore, uncontrolled studies of the use of KU in CFS patients showed improvement of disease manifestation. It would be important to follow KU treated CFS patients for levels of HHV-6 to assess its direct effects.

Third, the *in vivo* serum concentrations of KU, and the concentrations that develop within organs, are currently unknown; hence, it is impossible to draw clear clinical inferences from these *in vivo* studies.

Lastly, the active principal compound in KU is not known. Further studies are needed to purify the various KU fractions in order to find the one which has the most potent antiviral effects. This would reduce the dose *in vivo* to acceptable

levels and reduce the toxicity which may result from long term use of the drug.

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