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Inhibition of human adenoviruses by 1-(2′-hydroxy-5′-methoxybenzylidene)amino-3-hydroxyguanidine tosylate

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Abstract

Antiviral activities of four Schiff bases of aminohydroxyguanidine, designated ML1, ML4, ATL14 and LK11, were tested against human adenovirus types 5 and 8 (Ad5 and Ad8) in A549 cells by plaque reduction and virus yield reduction methods. Compound ML1 1-(2′-hydroxy-5′-methoxybenzylidene)amino-3-hydroxyguanidine tosylate gave the best therapeutic indices (TC_{50}/IC_{50}) of 27.2 and 17.8 for Ad5 and Ad8, respectively. Pretreatment of cells with ML1 did not affect the adsorption nor the penetration of virus. Ultrastructure studies showed that only the drug treated infected cells had unidentified irregular shaped electron dense structures that might be drug altered viral macromolecules that were not assembled into complete infectious virus particles. Since these compounds have metal chelating properties, their antiviral activity may involve the early IA (EIA) gene which encodes a viral protein of 289 amino acid which has a zinc finger moiety that is required for its transactivation activity.

Key words: Adenovirus; Antiviral; Schiff bases of aminohydroxyguanidine

1. Introduction

Adenoviruses infect epithelial cells of the eye, respiratory tract, intestinal tract, and adenoids and cause a variety of clinical manifestations. Although most of these infections are acute and self-limiting, they can become chronic, lasting for months to

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years. In recent years, it has been reported that severe and occasionally fatal adenoviral infections may occur in infants and young children (Uhnoo et al., 1984; Chiba et al., 1983; Yolken et al., 1982), and in immunocompromised patients (Shields et al., 1985; Stalder et al., 1977; Zahradnik et al., 1980).

Ocular adenovirus infections occur worldwide and frequently result in epidemics in the community and medical facilities. Epidemic keratoconjunctivitis, generally caused by adenovirus type 5, 8, 19 or 37, is a frequently occurring and highly infectious disease (Ford et al., 1987). Although (*S*)-HPMPC, (*S*)-HPMPA and 2′-norcyclic GMP were reported to have good antiadenoviral activities (Baba et al., 1987 and Gordon et al., 1991), currently there exists no antiviral treatment known to prevent or lessen the sequelae of adenovirus-induced diseases in the primary eye involved. Likewise, no treatment exists to protect against the spread of virus to the second eye or to other individuals. Recent reports (Gordon et al., 1992 and Tsai et al., 1992) have described animal models for evaluating the effect of potent experimental antiviral drugs against ocular adenovirus infections.

Hydroxyurea, hydroxyguanidine and thiosemicarbazone inhibit ribonucleotide reductase and have been reported to have anti-tumor as well as antiviral activities (Krakoff, 1975; Adamson, 1972; Sartorelli et al., 1971; Carter, 1973; Levinson et al., 1973). The substituted Schiff bases of aminohydroxyguanidine combine the structural features of these compounds (Fig. 1). A series of aminohydroxyguanidine compounds have been synthesized and tested for their biological activities in our laboratories (Tai et al., 1984; T'ang et al., 1985; Wang et al., 1990; Koneru, 1991). Antiviral activities for aminohydroxyguanidine have been reported against Rous sarcoma virus (T'ang et al., 1985), coronavirus (Wang et al., 1990), and herpes simplex virus type 1 (Lien et al., 1989). The contributions of different moieties of the molecule to the activities of these agents have been discussed (Lien et al., 1987a, 1987b, 1991). In this study, we report in vitro studies of the antiviral activities of Schiff bases of aminohydroxyguanidine against adenovirus types 5 and 8 (Ad5 and Ad8). Adenovirus type 5 is a common serotype that infects the ocular, respiratory and gastrointestinal systems, and type 8 is one of the more common serotypes that infects the eye and frequently causes epidemic keratoconjunctivitis.

2. Materials and methods

2.1. Cells and virus

Human lung tumor A549 cells from American Type Culture Collection, ATCC CCL185, were used as host cells for Ad5 and Ad8 as previously described (Tsai et al., 1992). Briefly, cells were grown at 37°C containing 10% of heat-inactivated fetal bovine serum (FBS). For plaque assays, the concentration of FBS was reduced to 2%.

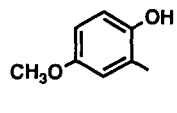
Adenovirus type 5 (WT300 from T. Shenk, Purdue University) and type 8 (ATCC) Stocks were prepared by inoculating A549 monolayers at a 0.01 multiplicity of infection with adsorption at room temperature and incubation at 37°C. When the cytopathic effect (CPE) involved approximately 75% of the cell monolayer, the

cultures were freeze-thawed, sonicated, centrifuged and titered by plaque assay. The virus was stored in aliquots at −70°C until used.

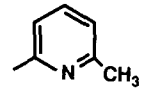
For the plaque assay method, A549 cells growing in 12-well tissue culture plates were inoculated with 10-fold dilutions of each sample (3 wells per dilution), and after the adsorption period the inoculum was removed and the monolayers were washed with Hank's Balanced Salt Solution (HBSS "1Xe" without Ca^{2+} and Mg^{2+}) to remove the unadsorbed virus and then overlaid with Ham's containing 0.75% methylcellulose. The monolayers were examined microscopically, and when plaques appeared clearly (after 6 days), the cultures were fixed with acetic acid/methanol (1:3), rinsed with water, stained with 1% crystal violet in methanol and counted.

2.2. Compounds

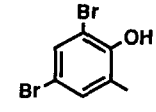
The structural formula of the substituted Schiff bases of aminohydroxyguanidine tosylate tested are shown in Fig. 1. The synthesis and chemical properties of these compounds were reported by Hui et al., unpublished results. All the compounds



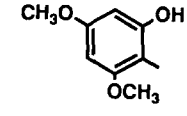
ML 1



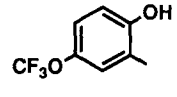
LT 2



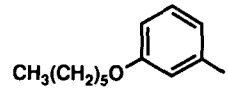
ATL 14



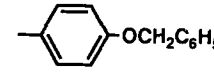
LK 3



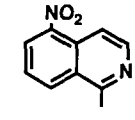
ML 4



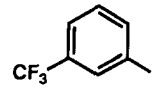
LT 7



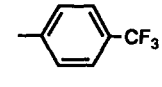
ATL 16



LK 11



LT 9



ATL 26

Fig. 1. Structures of the Schiff bases of 1-amino-3-hydroxyguanidine tosylate of the type **R**-NNHC(=NH)NHOH · $HSO_3C_6H_4CH_3$. The **R** groups substituted in the 1-position are as follows: compound ML1 2-hydroxy-5-methoxybenzylidene; compound ML4 2-hydroxy-5-trifluoromethoxybenzylidene; compound LT2 (6-methyl-2-pyridyl)-methylene; compound LT7 3-(hexyloxy)benzylidene; compound LT9 3-trifluoro-toluidene; compound ATL14 3,5-dibromo-2-hydroxybenzylidene; compound ATL16 4-benzyloxybenzylidene; compound ATL26 4-fluoromethoxybenzylidene; compound LK3 2-hydroxy-4, 6-dimethoxybenzylidene; compound LK11 (5-nitroisoquinol-1-yl)-methylene.

were initially dissolved in dimethyl sulfoxide (DMSO) and further diluted at 37°C with maintenance medium before use. The final highest concentration of DMSO used on the cultures was 0.16%. All compounds were tested at 0.1, 1, 10, 100 and 1000 μM. When necessary, additional drug concentrations were also tested (i.e., ultrastructure studies were performed at 0, 50 and 100 μM.

2.3. Cellular toxicity

Confluent monolayers of A549 cells in 24-well tissue culture plates were washed with HBSS and exposed to various concentrations of the compounds (3 wells were used for each concentration). Cells were examined daily by light microscopy for 6 days. Cytotoxicity was recorded when abnormal morphological changes were observed.

The effect of the compounds on cell proliferation was also evaluated quantitatively. When the cells were 60% confluent, triplicate monolayers were washed with HBSS and exposed to various concentrations of the compounds for 48 h incubation at 37°C. Monolayers were then dispersed into individual cells with trypsin and suspended in Ham's with 2% FBS and quantitated in a Coulter Counter. The expression used for drug toxicity was that concentration of a compound which reduced by 50% the cell count of treated cells compared to untreated cells, e.g., the TC_{50} of ML1 in A549 cells was 87 μM.

2.4. Antiviral activity

(a) Virus yield reduction assay. A549 cells were grown in 24-well plates in growth medium and incubated at 37°C. When the cell cultures became approximately 90% confluent, growth medium was removed and the cells were infected with virus at a multiplicity of infection (MOI) of 5 plaque-forming units (PFU) per cell.

After 1 h adsorption at room temperature, the inoculum was removed, monolayers were washed with HBSS, and maintenance medium containing various concentrations of compounds were added. Control cell (no drug or virus), drug control (no virus) and virus control (no drug) were included in each plate. When CPE was observed in the virus control, after 24, 48 and 72 h, the samples were freeze-thawed, sonicated, centrifuged and titered by plaque assay described above.

(b) Plaque reduction assay. Confluent A549 cells were grown in 12-well tissue culture plates and infected with approximately 100 PFU of virus stock per well. After one hour of virus adsorption at room temperature, overlay medium containing 0.75% methylcellulose without or with the test compounds ranging between 0.1 and 1000 μM were added to the infected cell monolayers. After 6 days of incubation at 37°C, the monolayers were fixed and stained as described above. Plaques were counted and the data plotted (antiviral concentration versus number of plaques) and the 50% inhibitory dose of the antiviral was determined from the graph.

The effect of drug treatment on virus inoculated cells was assessed by the plaque reduction method as follows:

(i) Uninfected 60% confluent A549 monolayers were preincubated with drugs for 24 h before inoculation, and treated with the same drug again post-inoculation; (ii)

the monolayers were preincubated with drugs for 24 h before inoculation but did not receive drug after inoculation; (iii) received drug treatment only after inoculation; a cell culture medium was given at the same time point of (i) and (ii) before inoculation. Inoculated monolayers were then incubated for six days before being fixed and stained for plaque counting.

2.5. Ultrastructure studies

The effect of compound ML1 on Ad8 infected-A549 cells was studied by ultrathin section transmission electron microscopy. ML1 (50 and 100 μM) was added to the inoculated monolayers after a one-hour virus adsorption with 5 PFU/cell, then incubated at 37°C for 24 h, fixed with half-strength Karnovsky's solution and processed for electron microscopy studies. Ultrathin sections were stained with uranyl acetate and lead citrate and examined in a Zeiss model 10 AEI (West Germany) transmission electron microscope at 60 Kv. Three sets of controls were studied in a similar fashion, i.e., cell control (no virus, no drug), virus control (no drug), and drug control (no virus, with drug).

3. Results

3.1. Plaque reduction assay and cytotoxicity

The concentration-response curves for compounds ML1, ML4, ATL14 and LK11 against Ad5 are presented in Fig. 2. These compounds prevented Ad5 plaque formation at μM concentrations. The IC_{50} values of ML1, ML4, ATL14 and LK11 with Ad5 were 3.2, 4.0, 5.0 and 3.2 μM, respectively. Similar plaque reduction data were observed in studies with Ad8 (i.e., IC_{50} values of 4.9, 2.4, 4.2 and 3.5 μM, respectively).

Based upon cytotoxicity curves (Fig. 2) which were presented as % cell growth reduction, ML1 appeared much less toxic than any of the other active compounds tested. The cytotoxicity curves of LK11, ATL14, and ML4 ended at 50 μM not because studies were terminated at this concentration but because concentrations beyond these points caused obvious morphological damage to the host A549 cells. Of the four potent antiviral compounds, ML1 had the most desirable therapeutic indices for Ad5 (27.2) and Ad8 (17.8) (unpublished data) in A549 cells and was selected for ultrastructure studies.

3.2. Virus yield reduction assay

ML1 (40 μM) gave a significant viral inhibition by reducing virus yield more than two log units at 24 h post-inoculation (Fig. 3). However, virus inhibition was less at 48 and 72 h post-inoculation. Since 24 h was the most sensitive point for measuring antiviral activity, virus yield assays of ML1, ML4, ATL14, and LK11 were all harvested at 24 h post-inoculation. Their relative antiviral activities are shown in Fig. 4. ATL14 reduced the virus yield most significantly, while LK11 was the least potent against Ad5 and Ad8 in A549 cells.

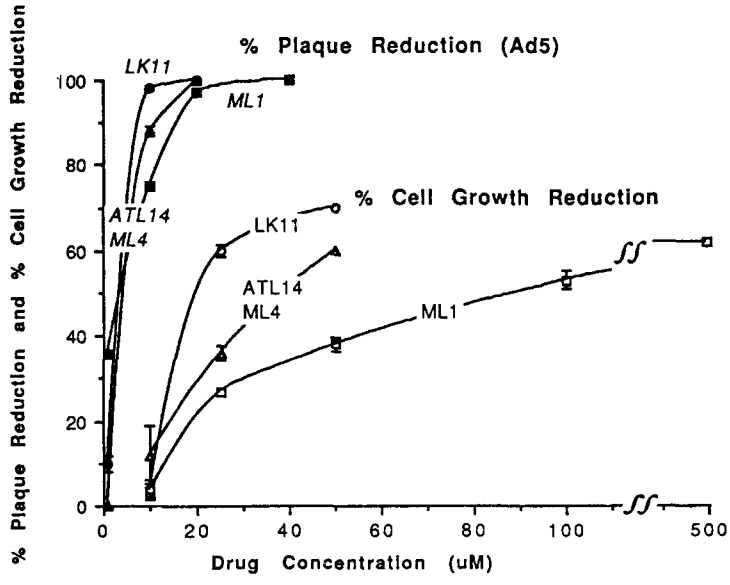


Fig. 2. Dose-response curves of inhibition against Ad5. Inhibition is shown as % Plaque Reduction on top left. Cytotoxicity on human lung tumor A549 cells is shown as % Cell Growth Reduction on top right. Drug concentration points represent 1, 10, 20 and 40 μM. Zero drug concentration point (not shown) was located at the origin. ATL14 and ML4 have very similar curves and are shown together. The IC_{50}s of ML1, ML4, ATL14 and LK11 are 3.2, 4.0, 5.0 and 3.2 μM, respectively. The TC_{50}s are 87, 36, 32 and 20 μM, respectively. ML1 has the highest therapeutic index of 27.2, while ML4, ATL14 and LK11's are 9.0, 6.4 and 6.3, respectively. The relative short Cell Growth Reduction curves of LK11 and of ATL14 and ML4 were because these compounds showed morphological damage to the host A549 cells at concentrations beyond 50 μM, whereas ML1 require concentrations beyond 500 μM. Plaque Reduction on Ad5: ■, ML1 treated; ▲, ML4 or ATL14 treated; ●, LK11 treated. Growth Reduction on A549 Cells: □, ML1 treated; △, ML4 or ATL14 treated; ○, LK11 treated.

3.3. Drug preincubation assay

Pretreatment of A549 cells with ML1 (i.e., 1, 5, 10, 25 and 50 μM) followed by drug removal prior to inoculation with Ad8 did not appear to interfere with virus adsorption or other steps in viral replication in that no plaque reduction was observed (Table 1). Pretreatment did not enhance significantly the antiviral activity of post-inoculation ML1 treatment. When ML1 containing medium was added to the inoculated cells 1 hour after virus adsorption, a reduction of Ad8 plaques was observed and ranged from 24% for 1 μM to 100% for the 50 μM concentration.

3.4. Ultrastructure studies

The morphology of A549 cells inoculated at 37°C with and without 50 and 100 μM of ML1 appeared identical. A thin-section of normal A549 cells with ML1 at 10000 magnification is shown on Fig. 5. Fig. 6 presents Ad8-infected A549 cells with clusters of virus particles at 24 h post-inoculation. Fig. 7 is an Ad8-infected A549 cell treated with ML1 (50 μM). Virus particles were present with unidentified

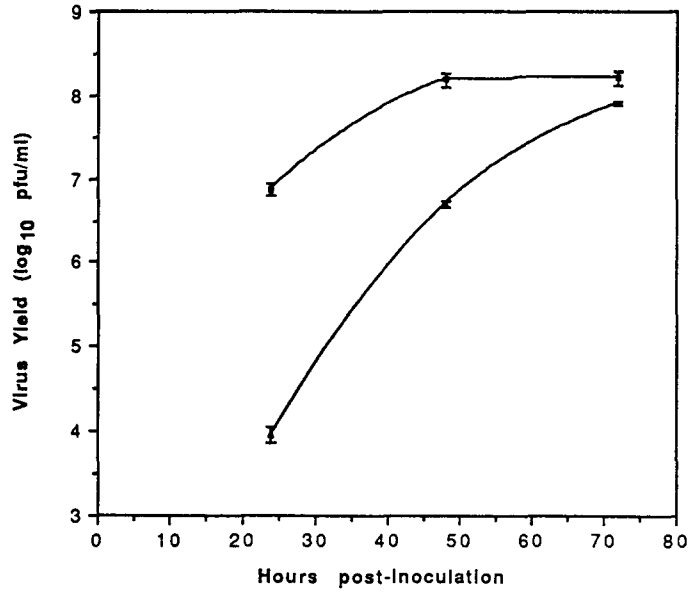


Fig. 3. Virus yield reduction assay of Ad8-infected A549 cells treated with ML1 (100 μM) after 1 h adsorption and harvested at 24, 48 and 72 h post-inoculation. The error bars are the standard deviation from two sets of experiments performed in triplicate cultures (i.e., 6 data points for each time period). □, Untreated Ad8-infected cells; ▲, ML1 treated Ad8-infected cells.

irregular shaped electron dense structures (see arrows on Fig. 7) which were unique to these drug treated virus infected cells. These structures were never observed in either ML1 treated normal A549 cells or untreated Ad8 infected cells. These struc-

Table 1
Effects of pretreating A549 cells with ML1 prior to Ad8 inoculation[a]

Conc. of ML1 (μM)	% Plaque reduction of Ad8 infection		
	Pretreated & treated[b]	Pretreated only[c]	Treated only[d]
1	31	0[e]	24
5	53	0	48
10	66	0	61
25	100	0	98
50	100	0	100

[a]Multiplicity of infection was 0.01.

[b]Pretreated and treated = uninfected A549 cells were preincubated with ML1 at the designated concentration for 24 h and treated with the same concentration of ML1 after infection with Ad8.

[c]Pretreated only = uninfected A549 cells were preincubated with ML1 for 24 h but were not treated with ML1 after infection.

[d]Treated only = uninfected A549 cells were incubated only with regular cell culture medium before infection but treated with ML1 after infection.

[e]No inhibition.

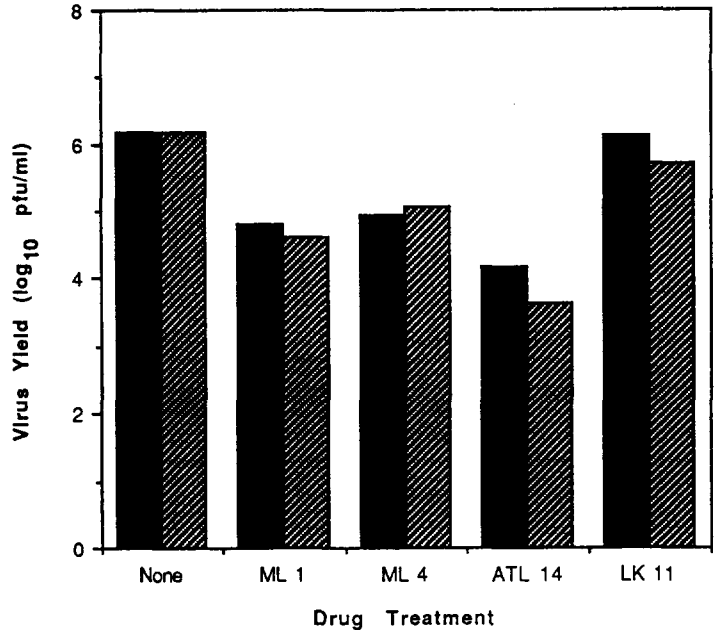


Fig. 4. Virus yield reduction assay of Ad5 and Ad8 infected A549 cells of ML1 (40 μM), ML4 (20 μM), ATL14 (20 μM) or LK11 (20 μM) after 1 h adsorption and harvested at 24 h post-inoculation. HELP , Ad5-infected; ■, Ad8-infected.

Table 2
Summary of the antitumor activities and ribonucleotide reductase inhibition of the substituted Schiff bases of aminohydroxyguanidine tested against Ad5 and Ad8

Compound	IC_{50} values in μM			
	Antiviral activity against		Antitumor activity[a-e]	Inhibition of ribonucleotide reductase
	Ad5	Ad8		
LT2	NI[e]	NI	42.1[a]	–
LT7	NI	NI	7.8[a]	–
LT9	NI	NI	15.1[a]	–
ATL14	5.0	4.2	12.7[b]	22[c]
ATL16	NI	NI	25.3[b]	33[c]
ATL26	NI	NI	9.2[b]	–
LK3	100	100	4.6[d]	–
LK11	3.2	3.5	2.95[d]	–

[a–d]These activities were previously reported. [a]Tai et al. (1984); [b]T'ang et al. (1985); [c]Cory et al. (1985); [d]Koneru (1991).

[e]NI = no inhibition using concentrations below TC_{50} values.

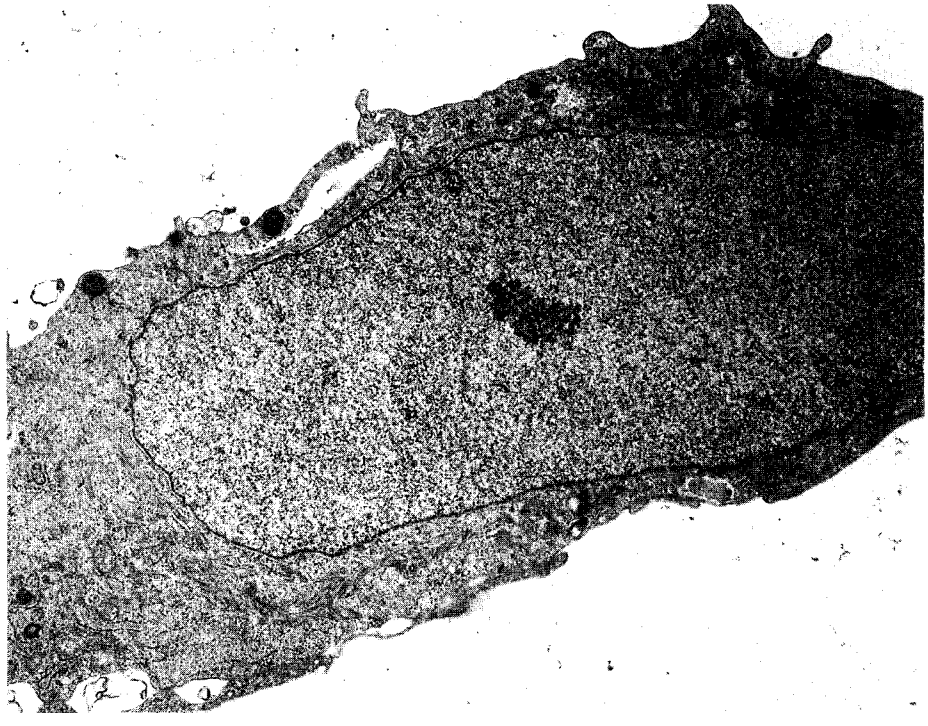


Fig. 5. A transmission electron micrograph (10 000 ×) of a thin-sectioned normal A549 cell after a 48-h exposure to 100 μM of ML1 at 37°C. The morphology of A549 cells before and after exposure to ML1 appeared identical.

tures could be 'drug altered' viral antigens that could not be assembled into complete infectious virus particles.

4. Discussion

Among the 23 Schiff bases of aminohydroxyguanidine tested reported elsewhere (Hui et al., unpublished results), ML1 is the most promising antiadenoviral agent. It consistently gave positive antiviral activity through both plaque reduction and virus yield reduction assay methods. ML1 showed the least cytotoxicity towards the A549 host cells among the other active compounds (i.e., ML4, ATL14 and LK11). The potent antiviral activity and minimal cytotoxicity suggest that ML1 may have clinical usefulness against adenovirus infection. The IC_{50} of ML1 is in the μM range and our unreported in vitro studies showed that ML1 is highly penetrable through conjunctiva and cornea to give on target treatment.

The methodologies used in our assessment of drug effects on cell morphology, viability and growth have many disadvantages such as subjectivity and lack of sen-

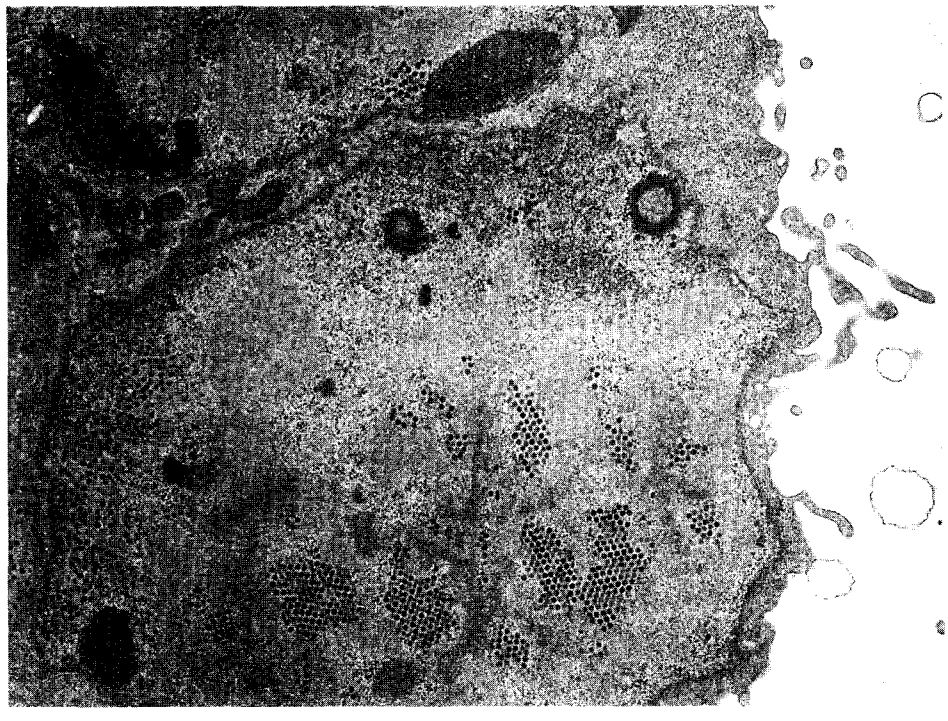


Fig. 6. Thin-sectioned Ad8-infected A549 cells (10000 ×). Virus particles appeared as crystalline lattices inside the nucleus.

sitivity and may have allowed for an under-estimation of toxicity. However, future studies of our most promising antiviral compounds will involve more detailed and stringent cytotoxic testing.

One of the reasons that we focused onto the screening of Schiff bases of aminohydroxyguanidine against adenoviruses was knowing the metal chelating capability of Schiff bases of aminohydroxyguanidine and the importance of zinc ions to the function of E1A gene product. E1A gene encodes a protein of 289 amino acids that has a zinc finger moiety that is required for its transactivation activity (Culp et al., 1988). E1A's expression is required for the transcription of all other early viral mRNAs. We propose that the transactivating ability of the 289 amino acid protein may be interfered with when the zinc is chelated by the aminohydroxyguanidine.

Adenoviruses are not the only viruses that Schiff bases of aminohydroxyguanidine can act on. They have been shown to have antiviral activities against Rous sarcoma virus (Tai et al., 1984; T'ang et al., 1985), coronavirus (Wang et al., 1990) and herpes simplex virus 1 (Lien et al., 1989). Table 2 summarizes the anti-tumor activities of some of these compounds (Tai et al., 1984; T'ang et al., 1985; Koneru, 1991) as well as inhibition of ribonucleotide reductase activity of ATL14 and ATL16 (Cory et al., 1985). Ribonucleotide reductase catalyzes the key step in the synthesis

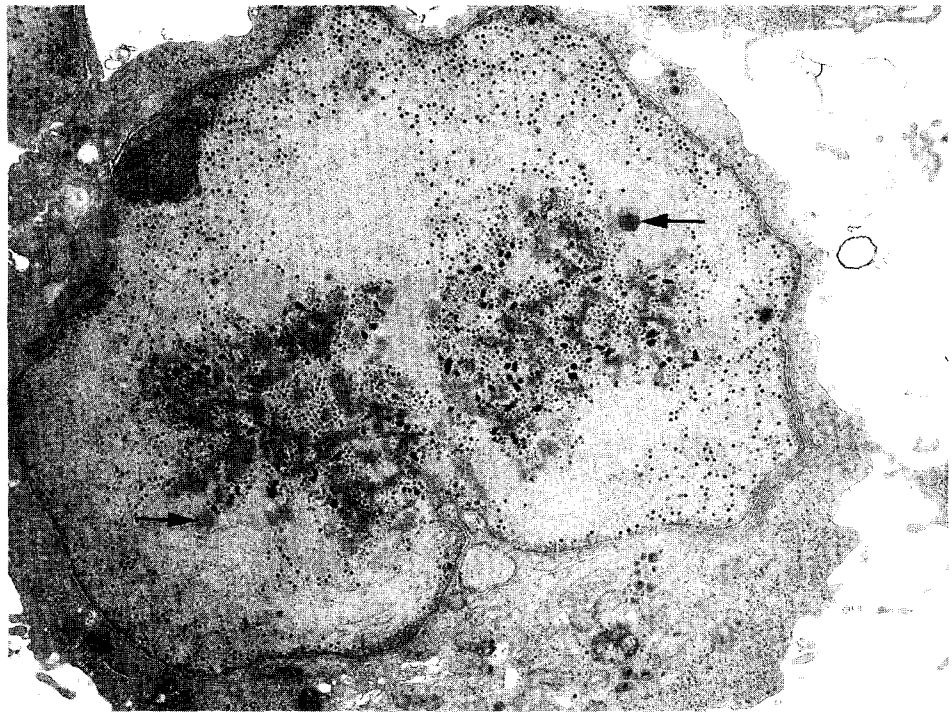


Fig. 7. Thin-sectioned Ad8-infected A549 cell treated with 50 μM ML1 (10000×). Virus particles are scattered inside the nucleus along with unidentified irregular structures (see arrows). This material could be modified viral macromolecules that could not be assembled into complete infectious virus particles.

of deoxyribonucleotides as precursors for DNA synthesis. It is possible that the antiviral activity of ATL14 against Ad5 and Ad8 is related to either or both the interference with the transactivating ability of the 289 amino acid protein and the inhibition of ribonucleotide reductase. Compounds LT2, LT7 and LT9 did not have any antiadenoviral activities, although they were shown by Tai et al. to have anti-tumor activities against cultured L1210 cells. Their IC_{50} against L1210 leukemic cell growth were 42.1 μM, 7.8 μM and 15.1 μM, respectively (Tai et al., 1985). T'ang et al. reported that the IC_{50} of ATL14, ATL16, and ATL26 against L1210 cell growth to be 12.7 μM, 25.3 μM, and 9.2 μM, respectively. ATL14 has shown potent anti-adenoviral activity in the present study but ATL16 and ATL26 have not shown any inhibition at all. In addition, both ATL14 and ATL16 were shown to have inhibition of the ribonucleotide reductase activity, their IC_{50} were 22 μM and 33 μM, respectively, but only ATL14 had antiadenoviral activity. Finally, for the LK series of compounds, LK3 had very low antiadenoviral activity but LK11 was potent. The IC_{50} values of their antileukemic activity against the CCRF-CEM/O cell lines were very similar (4.69 μM and 2.95 μM, respectively) (Koneru, 1991). There appears to be no obvious correlation between the antiadenoviral and the anti-tumor activity of

the Schiff bases of aminohydroxyguanidine with their ribonucleotide reductase inhibition activity.

ML1 did not inhibit viral replication at 48 and 72 h and this may be due to several factors. First, the kinetics of viral replication may be slowed in drug-treated cultures (Field, 1988). In this case, the apparent inhibition of yield is a function of time of assay of infectivity, and if left for a sufficiently long period, no inhibition of yield would be observed. Secondly, the antiviral drug stability of ML1 has not yet been established. An unstable compound might have less antiviral activity after an extended incubation period and multiple virus growth cycles, e.g., 48 or 72 h post-inoculation. Thirdly, the development of drug-resistance could be at least partially responsible for the failure of ML1 to inhibit virus growth at 48 h. In order to investigate this, both Ad5 and Ad8 were passed four different times in the presence of 40 μM of ML1. The IC_{50} of ML1 against Ad5 and Ad8 changed from 3.6 to 44 μM and 5.5 to 47 μM, respectively. This approximate 10-fold increase in IC_{50} values after multiple virus growth cycles in the presence of drug provides evidence of drug resistance.

Acknowledgements

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