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Inhibition of herpes simplex virus type 1 and adenovirus type 5 by heterocyclic Schiff bases of aminohydroxyguanidine tosylate

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Abstract

Eleven heterocyclic Schiff bases of aminohydroxyguanidine tosylate (SB-AHGs), compounds I–XI, were tested for antiviral activity against herpes simplex virus type 1 (HSV-1) and adenovirus type 5 (Ad 5) via plaque reduction and virus yield reduction assays. This work was undertaken to test the hypothesis that low molecular weight SB-AHGs (MW < 235 for the free SB) make better antiviral agents than high MW SB-AHGs (MW > 300). The plaque reduction assay method demonstrated that three compounds, I, VII and IX, had moderate activity against HSV-1, with 50% inhibitory concentration (IC_{50}) values of 38.0, 23.5 and 52.1 μM, respectively. Against Ad 5, compounds I, VIII and XI exhibited moderate activity, with IC_{50} values of 52.7, 19.3 and 5.1 μM, respectively. Among the compounds screened, compound I (1-[(3′-hydroxy-6′-methyl-2′-pyridyl)methylene]amino-3-hydroxyguanidine tosylate) was the most promising antiviral candidate, with selectivity indices (SI) of 10.2 (HSV-1) and 7.6 (Ad 5), respectively. Virus yield reduction assays indicated that compound I had less antiviral potency against HSV-1 than against Ad 5. The antiviral effects of compound I at a high input virus multiplicity of infection (MOI > 5) indicated that compound I had effective anti-adenoviral activity at 24 h post infection. This work demonstrated that some of SB-AHGs only have moderate antiviral activities against Ad 5 and HSV-1 viruses. In general, low MW SB-AHGs have low cytotoxicities to the host cells.

Keywords: Antiviral activity; Schiff bases of aminohydroxyguanidine tosylate; Plaque reduction assay; Virus yield reduction assay; Ribonucleotide reductase inhibitors

1. Introduction

Various Schiff bases of aminohydroxyguanidine tosylate (SB-AHGs) were developed in our laboratory as potential antitumor and/or antiviral

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[1] Taken in part from the Ph.D dissertation of A. Das, USC, School of Pharmacy, 1996.

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agents (Tai et al., 1984; T'ang et al., 1985; Wang et al., 1990; Koneru et al., 1993; Hui et al., 1994).

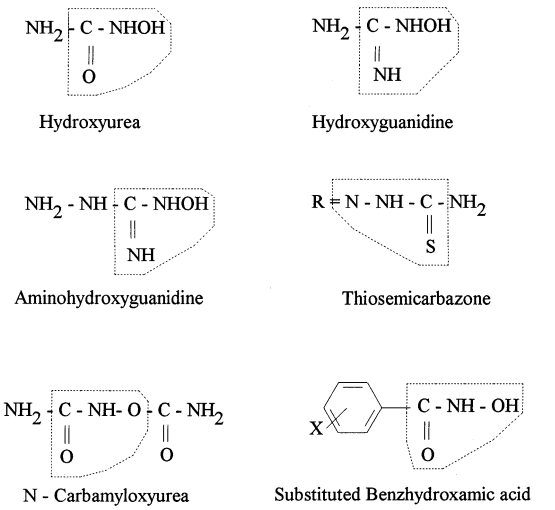

Fig. 1. Structural similarity of various ribonucleotide reductase (RR) inhibitors with aminohydroxyguanidine.

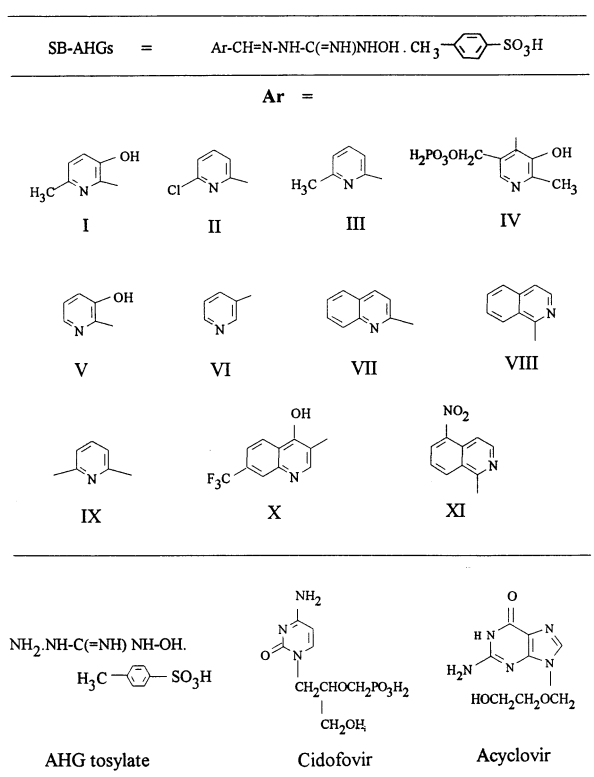

Fig. 2. Chemical structures of compounds studied.

Substituted SB-AHGs contain the essential pharmacophore of hydroxyguanidine (HG) and are structurally similar to hydroxyurea (HU), thiosemicarbazone (TSC) and *N*-carbamoyloxyurea as shown in Fig. 1.

Numerous potent and effective antiviral agents have limited use because of their cytotoxic effects on the host, the development of resistance by the viruses or both. Thus, current research in drug discovery is focused on developing novel, potent chemotherapeutic agents with target selectivity and low cytotoxicity, as well as combination chemotherapy that can enhance antiviral activity and overcome the problems of drug resistance.

Adenoviruses (Ad) are ubiquitous DNA viruses that are endemic in human populations around the world, sometimes causing epidemic outbreaks of respiratory and ocular infections. Forty-seven different serotypes of Ad have been characterized, one third of which are associated with a variety of human diseases (Horwitz, 1990). Although effective, oral enteric-coated vaccines can prevent diseases by Ad serotypes 4 and 7, these vaccines are used only in the military and are not licensed for administration to civilians. The common adenovirus serotypes 1, 2 and 5, which infect children, have not been attenuated. In spite of promising results achieved with some nucleoside analogues, such as [(S)-HPMPA], 2′nor-cyclic GMP, cidofovir (see Fig. 2) and ganciclovir, against adenoviral infections in in vitro (Gordon et al., 1991) and in vivo animal models (Trousdale et al., 1994), none of these are yet available for clinical use. Interferons may also have a beneficial effect (Mistchenko and Falcoff, 1987), but their large-scale synthesis is prohibitively expensive. Thus, a serious need exists for the development of specific agents to treat adenovirus-induced diseases in humans.

As previously mentioned, some SB-AHGs synthesized earlier in our laboratory had exhibited antiviral activities against Rous sarcoma virus, coronavirus and Ad 5 (Tai et al., 1984; Wang et al., 1990; Hui et al., 1994, respectively). Compounds with IC_{50}s of micromolar range against Ad 5 and Ad 8 have been found (Hui et al., 1994). Hence, it was postulated that synthesis of new SB-AHGs with proper structural features could yield potent, useful antiviral agents. Two indepen-

dent studies published earlier had indicated that heterocyclic systems, particularly the pyridine and isoquinoline derivatives, had superior biological activities to those of other aromatic and heteroaromatic systems (Blanz et al., 1970; Spector et al., 1991). It was also noted that of the SB-AHGs synthesized using benzaldehyde, salicylaldehyde, picolinaldehyde and isoquinolinaldehyde, single ring aromatic and heterocyclic compounds apparently had better antiviral activities than bulkier SB-AHGs. Thus, to confirm this hypothesis, two new heterocyclic SB-AHGs containing the picolinaldehyde ring system were synthesized, and screened along with nine other heterocyclic SB-AHGs for anti-adenoviral and anti-herpesviral activity.

2. Materials and methods

2.1. Compounds

Eleven heterocyclic SB-AHGs were screened for antiviral activity against Ad 5 McEwen strain (provided by Y.J. Gordon of The Eye and Ear Institute of Pittsburgh, Pittsburgh, PA) and HSV-1 McKrae strain (laboratory strain). The chemical structures of the compounds are shown in Fig. 2. Compounds I (1- [(3′-hydroxy-6′-methyl-2′-pyridyl) methylene] amino -3-hydroxyguanidine tosylate) and II (1- [(6′-chloro-2′-pyridyl) methylene] amino -3-hydroxyguanidine tosylate) were newly synthesized. The other compounds were previously synthesized in our laboratory [compound III (Tai et al., 1984), compounds IV and V (Wang et al., 1990), compounds VI–VIII (T'ang et al., 1985), and compounds IX–XI (Koneru et al., 1993)]. The synthesis and chemical properties of the new compounds have been reported elsewhere (Das et al., 1997). Cidofovir (Gilead Sciences, Foster City, CA) was used as a positive control against Ad 5, and acyclovir (Glaxo Wellcome, Research Triangle Park, NC) was used as a positive control against HSV-1. Stock solutions of all compounds except cidofovir were prepared as 0.1M solutions in DMSO; the stock compound of cidofovir was prepared fresh in double-distilled water and filter-sterilized. The final concentration of DMSO in the test solutions did not exceed 0.2%.

2.1.1. Cells and virus

Human lung tumor A549 cells [American Type Culture Collection (ATCC) Certified Cell Line (CCL) # 185] were used as host cells for plaque assays of Ad 5 McEwen strain. Vero cells (ATCC CCL # 81, African green monkey kidney cells) were used as host cells for plaque assays of HSV-1 McKrae strain. Cells were grown as monolayers in minimum essential medium (MEM) as previously described (Das et al., 1997). All cells were maintained at 37°C under a 5% carbon dioxide atmosphere. Ad 5 McEwen strain and HSV-1 McKrae strain were grown and titered in the respective cell lines as mentioned above. The virus stocks were stored in aliquots at −70°C.

2.2. Initial cytotoxicity studies

To determine cytotoxic concentrations of the compounds to be screened, confluent monolayers of A549 and Vero cells were exposed to various concentrations of the antiviral agents (I–XI, AHG. Ts and the positive controls) similar to the procedure used by Hui et al. (1994). Stock solutions of the compounds were serially diluted in growth medium to obtain final concentrations of 10^{-4} M to 10^{-8} M, and were tested for cytotoxicity in triplicate wells of 24-well tissue culture plates. A co-solvent DMSO control was run for each dilution tested and compared with cell controls to rule out any toxicity from the presence of DMSO. Cytotoxicity is based on abnormal morphologic changes observed in the cell monolayer (e.g. clumping, detachment or rounding up of cells). Results of cytotoxicity observed in the initial screening are shown in Table 1.

2.3. Plaque reduction assay and determination of 50% inhibitory concentration (IC_{50}) values

A549 and Vero cells were grown in 24-well tissue culture plates. Upon confluence the cells were infected with 100 plaque-forming units (pfu) of the virus per well, similar to the procedure used by Hui et al. (1994). Each experiment had cell

Table 1
Cytotoxicities and antiviral activities of heterocyclic Schiff bases of aminohydroxyguanidine

Compound	Cytotoxicity[a]	TC_{50}[b]	IC_{50} (μM)[c]	Selectivity index[d]	Molecular weight
I	−	388.6 (Vero cells), 400.7 (A549 cells)	38.0 ± 1.7 (HSV-1), 52.7 ± 5.7 (Ad 5)	10.2 (HSV-1), 7.6 (Ad 5)	209
II	−		>100 (both viruses)		213
III	−		>100 (both viruses)		193
IV	−		Inactive[e] (both viruses)		319
V	−		156.4 ± 33.2 (HSV-1)		195
VI	−		>100 (both viruses)		179
VII	+	78.8 (Vero cells)	23.5 ± 2.0 (HSV-1)	3.4	239
VIII	+	47.7 (A549 cells)	19.3 ± 5.0 (Ad 5)	2.5	239
IX	+	128.4 (Vero cells)	52.1 ± 4.7 (HSV-1)	2.5	279
X	+		Inactive[e] (both viruses)		313
XI	+	32.0 (A549 cells)	5.1 ± 1.0 (Ad 5)	6.3	274
Cidofovir	− (A549 cells)		6.5 (Ad 5)		
Acyclovir	− (Vero cells)		1.8 (HSV-1)		

[a] Cytotoxicity in both A549 and Vero cells except cidofovir (in A549 cells only) and acyclovir (in Vero cells only) at 100 μM concentration. − sign denotes no cytotoxicity; + sign denotes cytotoxicity.
[b] Required to inhibit growth of the host cells by 50%.
[c] Required to inhibit virus-induced cytopathogenicity by 50%.
[d] Ratio of TC_{50}/IC_{50}.
[e] No inhibition at 100 μM.

controls (no virus, no drug) and virus controls (no drug) in triplicate. After allowing virus adsorption at 37°C (2 h for Ad 5 and 1 h for HSV-1), the viral inoculum was removed and cells were washed with Hanks' balanced salt solution. Agar overlay medium containing various dilutions of the test compounds was then added to triplicate infected cell monolayers. Viral plaques could be clearly detected under the microscope 6 days postinfection with Ad 5 and 4 days postinfection with HSV-1. At this time cells were fixed with methanol: acetic acid (3: 1), then rinsed copiously with water to remove agar discs, stained with 1% crystal violet and rinsed again. Viral plaques were counted and data were plotted to obtain the dose response plots from which IC_{50} values were calculated. The dose response plots of compound I against Ad 5 and HSV-1 are illustrated in Fig. 3.

2.4. Virus yield reduction assay

The production of infectious virus particles (virus yield) on a time course basis, after infection with Ad 5 or HSV-1, was conducted with compound I, which was the most promising antiviral candidate. A549 cells were grown as monolayers in a 12-well tissue culture plate. Upon confluence,

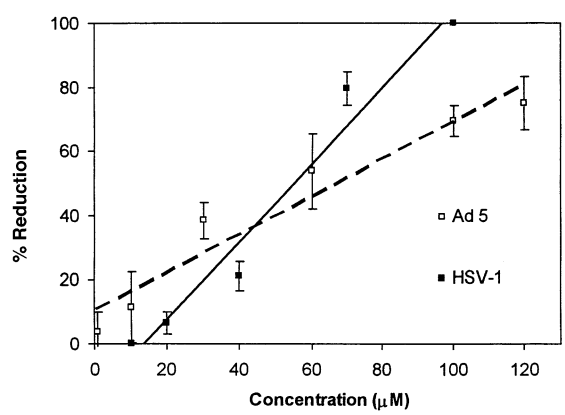

Fig. 3. Percent reduction of virus yield in the presence of different concentrations of compound I against Ad 5 in A549 cells ($Y = 0.59X + 10.47$, $r^2 = 0.94$) (open square) and against HSV-1 in Vero cells ($Y = 1.20X - 16.28$, $r^2 = 0.97$) (filled square).

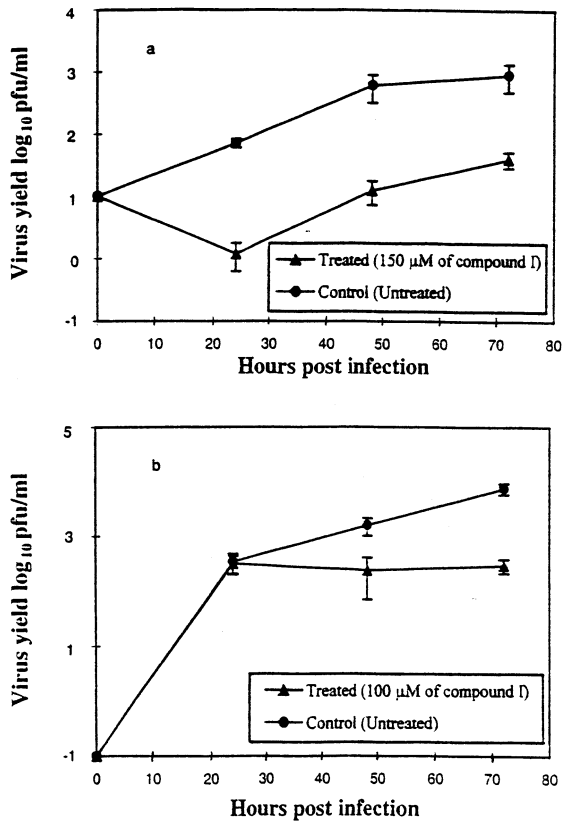

Fig. 4. (a) The virus yield of Ad 5 in A549 cells in the presence of compound I (150 μM) and (b) the virus yield of HSV-1 in Vero cells in the presence of compound I (100 μM) at 24, 48 and 72 h post infection.

the medium was removed and monolayers were inoculated with approximately 10^6 pfu of Ad5 virus per well. After incubating at 37°C for 2 h with frequent rocking, the viral inoculum was removed and monolayers were washed three times with Hanks' balanced salt solution. Six wells received 2 ml of growth medium containing 150 μM of compound I; another six wells received growth medium with no drug. The cell cultures were then incubated at 37°C in a 5% CO_2 humidified atmosphere. At periodic intervals (24, 48 and 72 h) after infection, duplicate samples from each group were collected separately by scraping the cells off the plate and freezing the cells and supernatant at −70°C. Samples were harvested at 72 h postinfection, freeze-thawed twice and centrifuged at 1500 rpm for 10 min to remove the cellular debris. The supernatants were serially diluted and titrated in A549 cell monolayers to ascertain the virus titers. For virus yield of HSV-1, a similar procedure was adopted using Vero cell monolayers in a 12-well plate inoculated with approximately 10^6 pfu per well and treated with 100 μM of compound I. The virus yield was plotted against hours postinfection, using Microsoft Excel Ver 5.0 (Microsoft Excel, 1993). The results are shown in Fig. 4.

2.5. Determination of 50% toxic concentration (TC_{50}) and selectivity

2.5.1. Selectivity Index (SI) values

The effect of compounds on cell growth was also evaluated quantitatively to determine TC_{50} values in A549 and Vero cells. The procedure was a modification of the method described by De Clercq et al. (1987). Only the TC_{50} values of compounds I, VII–IX and XI were determined. Using the growth medium, selected compounds were serially diluted from the stock to obtain concentrations ranging from 0.01 μM to a maximum of 300 μM (in the case of ADL 1), based on results of preliminary cytotoxicity studies. The A549 and Vero cells were grown in 24-well tissue culture plates to approximately 70% confluency. Triplicate wells were then exposed to various concentrations of the drugs in growth medium, while three cell control wells received growth medium with no drugs. After 2 days of incubation at 37°C, when the monolayers were nearly confluent, medium was removed, the monolayers were trypsinized, and the cells counted using a Coulter counter. Data were converted to determine % live cells, and dose response curves were plotted to determine TC_{50} using the same program used for determining IC_{50} values. Selectivity index, a measure of the margin of safety of the anti-viral agent, was then determined as a ratio TC_{50}/IC_{50}. Results are shown in Table 1.

3. Results

Initial cytotoxicity experiments in both A549 and Vero cells revealed compounds I–VI to be nontoxic to confluent cells at 100 μM (the maximum concentration tested in most cases) while compounds VII–XI were cytotoxic (Table 1), as observed by abnormal morphologic changes in the cell monolayers (e.g. clumping, detachment or rounding up of cells). Thus, plaque reduction assays for compounds VII–XI were conducted at concentrations below 100 μM.

Only compounds I, VIII and XI inhibited Ad 5 at concentrations < 100 μM; but compounds VIII and XI were also cytotoxic to host A549 cells at concentrations > 50 μM. The IC_{50} values against Ad 5 were 52.7 μM (compound I), 19.3 μM (compound VIII) and 5.1 μM (compound XI), respectively. Compounds I, VII and IX were found to have moderate activity against HSV-1 with IC_{50} values of 38.0, 23.5 and 52.1 μM, respectively. It can therefore be concluded that some of the heterocyclic SB-AHGs had only moderate anti-adenoviral and anti-herpesviral activity. Only compound I demonstrated activity against both viruses, and the dose response plots are shown in Fig. 3. Inhibition of both viruses by compound I followed a concentration-dependent manner, and 75 and 100% reductions against Ad5 and HSV-1 viruses were achieved at 120 and 100 μM, respectively. The IC_{50}, TC_{50} and SI values of the five compounds of interest are summarized in Table 1.

Yield reduction assays were carried out for compound I, the most promising antiviral candidate against Ad 5 (IC_{50} of 52.7 μM vs. IC_{50} of 6.5 μM for cidofovir as a positive control) and HSV-1 (IC_{50} of 38.0 μM vs. IC_{50} of 1.8 μM for acyclovir as a positive control). A higher concentration (approximately three times the IC_{50}) of the compound was used because compound I was nontoxic to A549 and Vero cells at concentrations of up to 150 μM and because a higher multiplicity of infection of the viruses was being used in these assays. Approximately 10^6 pfu of each virus was used to infect the cell monolayers instead of the 100 pfu that is typically used in the plaque reduction assay. The advantage of this assay is its usefulness in studying the effect of an antiviral agent on the virus growth cycle at a high-input multiplicity of the challenging virus.

The yield reduction assay against Ad 5 (Fig. 4a) indicated that compound I inhibited adenovirus multiplication in the early stages of infection or within 24 h and that it maintained a lower yield of virus particles compared to the virus control for a duration of at least 3 days (72 h). The yield of infectious virus was maximally reduced by about 2 logs at 24 h post infection, after which there was an apparent increase in the number of virus particles in the treated cells at almost the same rate as in the control cells. These results resemble an earlier study with Ad 8 and structurally similar compounds (Hui et al., 1994), and support the observation that SB-AHGs probably inhibit an early stage of virus replication. However, in the HSV-1 virus yield reduction assay (Fig. 4 b), there was no apparent reduction in yield with compound I 24 h post infection; but there was a modest reduction (sevenfold) at 48 h and a reduction of 25-fold (more than one log unit) at 72 h. This indicates that SB-AHGs have different pharmacokinetic profiles against HSV-1 and Ad 5.

4. Discussion

Among the 11 heterocyclic SB-AHGs tested for antiviral activities, compound I was the most promising antiviral agent, with activity against both Ad 5 and HSV-1. Compound I also had the least cytotoxicity toward host A 549 cells ($TC_{50} =$ 400.7 μM) and Vero cells ($TC_{50} = 388.6$ μM) compared to other compounds with moderate antiviral activities, namely, VII and IX (against HSV-1) and VIII and XI (against Ad 5). Compound I may thus have the potential for development as a broad-spectrum antiviral agent.

From the initial screening, 11 compounds can be divided into two groups with respect to morphological changes in cells, namely group I (noncytotoxic, compounds I–VI) and group II (cytotoxic, compounds VIII–XI). All five SB-AHGs with MW < 235 (compounds I–III, V, VI) in group I were found to be noncytotoxic at the 100 μM concentration in both cell lines. Only

one (compound IV) of six SB-AHGs in group I have MW > 235, but still noncytotoxic at 100 μM concentration. In contrast, all five group II SB-AHGs with MW > 235 (compounds VII–XI) are cytotoxic at 100 μM (Table 1). The present work further supports our hypothesis that smaller compounds (MW of approximately 170–220 for the free SB) are generally less cytotoxic and potentially better antiviral agents than the bulkier compounds (Wang et al., 1990). Compounds VII–XI (with MW's of 235–315 for the free Schiff bases) were highly cytotoxic to the host cells, and thus, they may have potential as effective antitumor agents since cytotoxicity against transformed cells is an important feature of antitumor activity. In fact, earlier studies with these SB-AHGs (compounds VII–XI) did demonstrate potent antineoplastic activities for some compounds (T'ang et al. 1985; Koneru et al. 1993). Compounds VII and VIII had IC_{50} values of 6.8 and 3.3 μM, respectively, in cultured L1210 murine leukemia cell lines (T'ang et al., 1985). Compounds IX and XI had potent cytotoxicities in CCRF-CEM/0 human lymphoblastic leukemia cell line, with IC_{50} values of 7.1 and 3.0 μM, respectively (Koneru et al., 1993).

The antitumor activities of hydroxyurea, hydroxyguanidine, thiosemicarbazone and Schiff bases of aminohydroxyguanidine are due to their ability to inhibit the enzyme ribonucleotide reductase (RR), a key enzyme required for de novo DNA synthesis in all cells (Cory et al., 1985). However, the antiviral activities of substituted SB-AHGs may or may not be due to RR inhibition alone since these compounds are effective against DNA viruses (adenovirus, Hui et al., 1994), RNA viruses (coronavirus or murine hepatitis virus, MHV, Wang et al., 1990) and the RNA tumor viruses (Rous sarcoma virus, RSV, Tai et al., 1984). An earlier study showed that while the antitumor activities of SB-AHGs correlate significantly with RR inhibition, no such correlation exists between RR inhibition and antiviral activity in a quantitative structure-activity relationship (QSAR) analysis of SB-AHGs against the growth of L1210 cells and transformation of chick embryo fibroblasts by RSV in vitro (Lien et al., 1989). However, inhibition of RR as a mechanism of antiviral activity cannot be ruled out since mechanistic studies of inhibition of virus-induced RR by SB-AHGs have not been conducted.

Wang et al. (1990) had proposed that both the antitumor and antiviral activities of SB-AHG are due to their potential for chelating divalent metal ions that are important cofactors for various enzymatic activities. It was also postulated that the probable mechanism of anti-adenoviral activity of SB-AHGs was chelation of the zinc ion (Zn^{++}) serving as part of the Zinc finger motif that is present in one of the two major proteins encoded by the immediate early region (E1A) gene product of the adenovirus (Hui et al., 1994). The expression of the E1A gene product is essential for the transcription of all other early viral mRNAs.

In the arena of antiviral chemotherapy, it is desirable to achieve selective toxicity toward the virus with minimum toxic effects to the host cells. As mentioned earlier, rapid development of resistant strains of virus is posing a serious threat to treatment with the available antiviral agents. Further research in this field is still warranted to develop more effective antiviral agents.

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