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Anti-HSV activity of serpin antithrombin III

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Abstract

Natural serine protease inhibitors (serpins) elicit sensing of a microbial cell intruder and activate an intrinsic cellular immune response in HIV and HCV infected cells. Here, we demonstrate *in vitro* inhibition of HSV with serpin antithrombin III (ATIII) early during infection pointing towards inhibition of an entry event. We also found reduction of mortality from 90% to 40% in an abrasion mice model demonstrating a strong reduction of infection *in vivo*. Our data also indicated that this treatment might be suitable for drug-resistant viruses since high inhibition of an acyclovir-resistant HSV-1 strain was found. Thus, an ATIII tropical treatment might be used for immunocompromised patients where prolonged treatment leads to drug resistant HSV-1 strains. Understanding how ATIII regulates HSV-1 infections may reveal new avenues for therapeutic interventions.

Keywords

Antithrombin III; HSV; serine protease inhibitor; serpin; acyclovir-resistant HSV-1; mice abrasion model

I. INTRODUCTION

The long-term treatment of herpesvirus infections with current antivirals especially in immunocompromised hosts leads to the development of drug-resistant viruses. Nearly all currently available antivirals finally target viral DNA polymerases, therefore virus resistant to one drug often shows cross-resistance to other drugs. In addition, nearly all the current antivirals show various kinds of side effects or poor bioavailability. The prevalence of acyclovir (ACV)-resistant HSV among immunocompromised patients is approximately 5%, and reaches 14% among bone marrow transplant recipients [1–3].

The increasing number of immunocompromised individuals, particularly AIDS and transplant patients, has driven the need for improved antiviral agents to treat diseases caused

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CONFLICT OF INTEREST STATEMENT
We have no conflict of interest to declare.

by herpesviruses. This evidence highlights the need for developing new antivirals for herpesviruses that have a different mode of action. Novel antiviral drugs that target cellular proteins instead of viral targets have the advantage that targets are immutable [4]. This approach might reduce the occurrence of resistant viruses.

However, the inhibitors of cellular proteins essential for viral replication might be constrained by significant side effects. Therefore, we utilized a unique search strategy to limit possible side-effects. We searched for an anti-viral factor in a disease population which did not get sick in spite of being infected with a life threatening virus and being untreated. We reasoned that such an intrinsic anti-viral factor should show a good toxicology profile. We isolated the serine protease inhibitor (serpin) antithrombin III (ATIII) as one factor responsible for HIV-1 inhibition in $CD8^+$ T cells of long-term non-progressors [5, 6], a patient population infected by HIV-1 for more than 10 years but which do not progress to AIDS. Testing ATIII against 18 pathogenic human viruses and sub-strains we further found that ATIII had a broad-spectrum anti-viral activity for HIV-1, HCV and HSV [7].

Herpes virus is spread through direct skin to skin or mucous membrane contact. Transmission likely occurs during symptomatic reactivation of latent virus. HSV-1 is typically acquired orally during childhood, but may also be sexually transmitted with approximately 30% of genital herpes in the U.S. caused by HSV-1 [8]. HSV-1 infection provides some immunity against subsequent infection with HSV-2. Symptoms from the primary infection with HSV are usually more severe than subsequent outbreaks due to lack of an antibody response. Latent infection of HSV typically in the neural ganglia results from 1.) the ability of HSV to evade the immune system through interference with MHC class I presentation of antigen on the cell surface by blocking the TAP transporter through secretion of ICP-47 and 2.) expression of latency associated transcript (LAT) RNA that regulates the host cell genome and interferes with natural cell death mechanisms [9].

In the first year of infection with HSV-1, the rate of asymptomatic shedding is approximately 5% without observable symptoms and the recurrence rate is 50% [10]. Though it has not yet been explained, the rate of viral shedding decreases over time. The frequency and severity of outbreaks depends on three factors: 1.) how well the person's immune system can control the infection, 2.) how long the person has been infected, and 3.) whether the virus established latency in its site of preference with less severe symptoms occurring when HSV-1 persists outside the trigeminal ganglia. Current systemic treatments for HSV-1 which inhibit the HSV DNA polymerase include ACV and its prodrug, valacyclovir, and famciclovir, the prodrug of penciclovir. FDA approved topical treatments include ACV, penciclovir and docosanol. Treatments reduce virus replication but do not cure the infection.

Our product development strategy utilizes a topical microbicides based on a critical component of the coagulation cascade which is also part of an early innate immune response [11]. Our studies aim to develop a topically applied product which will reduce or eliminate the severity of symptoms during reactivation episodes and reduce the transmission of HSV from virus shedding. We demonstrated that after activation of the serine protease activity through heparin the activated hep-ATIII inhibits HSV-1 and HSV-2 [7]. It is possible that

ATIII might be used as a single topical agent or in combination with other antiviral agents against HSV to target multiple steps of virus replication at the same time.

II. METHODS

Ethical Statements

All animals were housed and maintained in accordance with the Guide for the Care and Use of Laboratory Animals [12], and all studies and procedures were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of University of Alabama at Birmingham. The University of Alabama follows NIH guidelines for animal handling and has an Animal Welfare Assurance on file with the Office for Protection of Research Risks. This institution maintains full accreditation from Association for Assessment and Accreditation of Laboratory Animal Care.

Viruses

$HSV\text{-}1_{FL}$, $HSV\text{-}1_{HF}$, $HSV\text{-}1_{KOS}$, $HSV\text{-}1_{MacIntyre}$ $HSV\text{-}1_{E\text{-}377}$ and the ACV-resistant $HSV\text{-}1_{DM2.1}$ were described earlier [13–17].

Production of hep-ATIII

Recombinant human ATIII was produced in transgenic goats by GTC Biotherapeutics (Framingham, MA). These transgenic animals express human ATIII in their mammary glands and secrete it into their milk. ATIII was purified from goat milk through clarification through a 500-kDa tangential flow membrane filtration unit, captured and then eluted through a heparin affinity chromatography column. It was further purified by anion exchange chromatography and hydrophobic interaction chromatography as described earlier [18]. ATIII was activated with equal amounts (w/w) of heparin sodium (Polysciences, Warrington, PA, cat. no. 01491, 3–5 kDa fraction) overnight at 37 °C to form a non-covalent ATIII-heparin complex. Unbound heparin was then removed by Sephacel 100 ÄKTA FPLC (GE Health Care Life Sciences, Piscataway, NJ) at 1 ml/min. Protein preparations resulted in less than 5% (w/w) free heparin measured by FPLC with refractive index detection as described earlier [19]. Protein purity was more than 99% as determined by dodecyl sulfate-polyacryl-amide (SDS-PAGE) gel electrophoresis and silver staining using a Bio-Rad kit (Bio-Rad Life Science, Hercules, CA), or by C4 high-performance liquid chromatography (HPLC). Molecular weight was 60 kDa as compared to a low-range protein molecular weight marker (Bio-Rad) [20].

Formulation of the hep-ATIII in carbopol gel vehicle

Hep-ATIII (20 mg/ml in PBS) was stable > 30 days at 4° C. This solution was mixed at a 1:1 ratio with a 1.5% carbopol gel for the application. Mixture was loaded into 1-cc syringes and stored refrigerated for the duration of the study. Five percent (w/w) ACV (Zovirax®, GlaxoSmithKline Pharmaceuticals) in polyethylene glycol base was used as a control and maintained at room temperature.

Cells

Human foreskin fibroblast (HFF) cells were prepared from human foreskin tissue. The HFF cells were expanded through serial passages in standard growth medium of MEM with Earl's salts supplemented with 10% (v/v) FBS and antibiotics. The cells were passaged routinely and used for assays at or below passage 10. For comparative studies we also used Vero (African green monkey kidney) cells.

Determination of *in vitro* inhibitory efficacy by cytopathic effect (CPE) reduction assay

Low passage (3–10) HFF cells were trypsinized, counted, and seeded into 96 well tissue culture plates in 0.1 ml of MEM supplemented with 10% FBS. The cells were then incubated for 24 hours at 37 °C. The experimental drug was added in triplicate wells. Media alone was added to both cell and virus control wells. The drug was then diluted serially 1:5. The plates were then incubated for 60 min and 100 μl of a virus suspension was added to each well, excluding cell control wells which received 100 μl of MEM. The plates were then incubated at 37°C in a CO_2 incubator for three days. After the incubation period, media was aspirated and the cells were stained with crystal violet in formalin for 4 hours. The stain was then removed and the plates were rinsed until all excess stain was removed. The plates were allowed to dry for 24 hours and the amount of stain as measure for CPE in each well was determined using a BioTek Multiplate Autoreader. The 50% effective concentration (EC_{50}) and the 50% cytotoxic concentration (CC_{50}) values were determined by comparing drug treated and untreated cells. To determine if each compound has sufficient anti-viral activity that exceeded its level of toxicity, a selectivity index (SI) was calculated according to CC_{50}/EC_{50}. An inhibition that had a SI of 10 or greater was considered to have useful anti-viral activity.

Determination of *in vitro* inhibitory efficacy by plaque reduction assay

Monolayers of HFF cells were prepared in six-well plates and incubated at 37°C for 2 days to allow the cells to reach confluency. Media was then aspirated from the wells and 0.2 ml of virus was added to each of three wells to yield 20–30 plaques in each well. The virus was allowed to adsorb to the cells for 1 hour and the plates were agitated every 15 minutes. Compounds were diluted in assay media consisting of MEM with Earl's salts supplemented with 2% (v/v) FBS, and antibiotics. Solutions ranging from 0.1 to 300 μM were added to duplicate wells and the plates were incubated for various times, depending on the virus used. The monolayers were then stained with 1% crystal violet in 20% methanol and the unbound dye removed by washing with dH_20. For all assays, plaques were enumerated using a stereomicroscope and the concentration of compound that reduced plaque formation by 50% (EC_{50}) was interpolated from the experimental data. Cytotoxicity was measured in parallel by XTT staining.

Time addition experiments

To test if inhibition was at the entry stage or post-entry HFF cells were infected as described above and drug was added at the same time or 2 hours after infection.

HSV-1 *in vivo* inhibition test

Groups of 10 SKH-1 Elite females (Charles River Laboratories), approximately 5–6 weeks old at study initiation were randomly assigned to three groups for efficacy.

Oral antibiotics (trimethoprim-sulfamethoxazole or Baytril) were added to water bottles 24 hours prior to viral inoculations. Mice were anesthetized with ketamine/xylazine. Abrasions were performed carefully with an engraving bit attached to a hand held Dremmel Tool at a depth not sufficient to cause bleeding. The abraded area had a triangular shaped area over the nasal bones from the nose leather to the eyes. The abraded area was swabbed for 10 seconds with a 1×10^5 pfu/ml HSV-$1_{E\text{-}377}$ in MEM soaked Dacron swab.

Mice were treated topically three times daily with hep-ATIII gel for 7 days beginning 24 hours post viral inoculation. Doses of the gels were applied with a Dacron swab using approximately 30 μl/mouse and treatments were applied after viral swabs and clinical observations of lesion symptoms were performed. ACV treatments started after 24 hours, three times daily for 7 days. To determine the effect of treatment on the development and severity of lesions, severity of symptoms was graded on a 0.0 to 5.0 scale in 0.5 increments (Table I). The presence or absence of lesions and severity of lesions was recorded daily beginning day 1 through day 21 post-challenge.

Swabs for viral quantitation of inoculation sites was taken at days 2–8 and 10 prior to first daily treatments. Samples will be frozen at −70°C until titrated on HFF cells.

Animals were observed daily for signs of systemic viral disease. Groups of mice treated with antivirals were compared to vehicle-treated groups for statistical evaluation.

Measurement of virus titer

Swab samples were collected with a pre-moistened Dacron swab. The swab samples were placed in 1 mL of maintenance medium and stored frozen at −80 °C until virus titration using the plaque assay. HFF cells were seeded into a 96-well tissue culture plate at 1×10^5 cells/well and incubated at 37 °C in a humidified atmosphere with 5% CO_2 for 3 days. After cells formed a monolayer with approximately 70% confluency, the growth medium was removed, and samples in triplicate were serially diluted 10-fold using an automated liquid handler. The plates were incubated for 5 days at 37 °C. in a 5% CO_2 incubator. The CPE was read using a microscope and HSV-1 titers were calculated as tissue infection dose 50% ($TCID_{50}$) using the Reed & Muench method of calculation.

Statistical Evaluation

Topical lesion data are presented as means and the standard deviation (SD). In the efficacy study, infection rates, peak virus titers, virus titer–day areas under the curves (AUC), peak lesion scores and lesion score–day AUC between drug and vehicle treated groups were compared using the Mann–Whitney *U*-Rank Sum Test. Mortality rates were analyzed by Fisher's exact test and checked for a 21-day interval. The mean day of death (MDD) data were analyzed by Mann–Whitney *U*-Rank Sum Test. *P*-values of 0.05 or less were considered to be statistically significant.

III. RESULTS

Comparative *in vitro* anti-viral activity of Hep-ATIII and ACV

We expanded on earlier *in vitro* HSV-1 inhibition experiments and performed additional experiments in Vero cells and HFF and compared the hep-ATIII activity to the current HSV-1 treatment (Table II). We found that hep-ATIII had routinely a higher efficacy (up to 10 fold) than the ACV compare in our infection models. We found that the SI was > 10 for all of our experiments. Thus, no cytotoxicity was found for the concentrations tested. This demonstrated that hep-ATIII had good *in vitro* anti-viral activity independent on the cell type used.

Identification of virus life cycle inhibition step and inhibition capacity for ACV-resistant strain

In order to test if hep-ATIII inhibits at the stage of entry or post-entry we added the compound at the same time with the virus to the cells or 2 hours post infection. We used two HSV-1 strains for our experiments E-377 and the ACV-resistant DM2.1; both strains were strongly inhibited at EC_{50} of 0.54 and 0. 52 μM and EC_{90} of 3.9 and 1 μM, respectively. We found that the majority of inhibition was at the entry level since inhibition was lost when hep-ATIII was added 2 hours after infection (EC_{50} > 5 μM).

Hep-ATIII treatment might be used to treat ACV-resistant HSV-1 in immunocompromised patients. Therefore, we tested hep-ATIII activity for DM2.1, an isolate which is not inhibited by ACV even at concentrations > 100 μM. We found that the hep-ATIII anti-viral activity was similar to that found for ACV-sensitive strains.

Effect of hep-ATIII on mortality and on mean day of death (MDD)

We utilized SKH-1 mice to measure inhibition of mortality. Topical treatment with vehicle, ACV and hep-ATIII were applied 3 times daily starting at 6 AM for 7 consecutive days daily in 6 hours intervals beginning 24 hours post viral inoculations. We used an amount of virus for infection which led to at least 90% mortality and a MDD 7.4 days in the vehicle control (Table III). The Zovirax® gel control reduced mortality 100% in our animal model. Hep-ATIII used at 10 mg/ml reduced mortality 2.3-fold (P<0.01) but MDD was 7.5±0.6 and unaffected.

Effect of hep-ATIII effect on lesion day AUC, mean peak lesion score, virus titer day AUC and mean peak virus score

Hairless SKH-1 mice allow a standardized scoring to measure the HSV-1 lesion symptoms (Table I). All mice developed lesions. Lesion day AUC values were lowest in the ACV treated group (4.1) compared to the vehicle treated group (17.4) or the hep-ATIII treated group (12.4) although all these treatments did not achieve statistical significance (Table IV). The comparisons of mean peak lesion scores were not statistically different: The ACV treated groups had the lowest value (1.3±0.5) compared to the vehicle group (3.0±0.2) or hep-ATIII group (2.9±0.3) (Table IV).

We then measured the titer from swabs of the different treatment groups to measure if there is a reduction in virus shedding. We found that all swabs were positive for the HSV-1. We found that hep-ATIII treatment did decrease the virus titer day AUC values from 36.8 to 29.5 although reduction was not significant ($P = 0.09$). ACV treatment reduced virus titer day AUC values to 17.2 ($P < 0.01$). Hep-ATIII treatment did not reduce the mean peak virus score AUC values. Thus, hep-ATIII treatment did not decrease virus shedding (Table V).

IV. DISCUSSION

Nearly all currently available anti-HSV drugs finally target viral DNA polymerases. Furthermore, virus resistant to one drug often shows cross-resistance to other drugs [21]. In addition, nearly all current anti-HSV drugs show various kinds of side effects or poor bioavailability [22]. New treatment options are urgently needed.

We found that *in vitro* hep-ATIII was multiple-fold more effective than ACV. We also found that *in vitro* hep-ATIII was efficacious for ACV-resistant HSV. Therefore, our results indicated that hep-ATIII might be a possible second line treatment in immunocompromised patients due to AIDS, organ transplantation, or cancer chemotherapy. Long-term treatment in these immunocompromised hosts leads to the development of drug-resistant viruses [22]. ACV-resistant mutants were responsible for HSV pneumonia, progressive whitlow, meningoencephalitis, and mucocutaneous dissemination in AIDS patients [23–27].

One strategy to prevent the occurrence of resistant viruses is to target immutable host cell factors necessary for entry, viral replication or maturation. ATIII was found to block HIV and HCV through a not completely understood intracellular mechanism, although the anti-inflammatory effect of ATIII seems to play a major role [7, 20]. Serpins were found also to block HIV-1 inhibition at the entry level, although to a lesser extent [28]. Surprisingly, we found that hep-ATIII was only inhibiting HSV-1 at an entry step. It is possible that the cell types used have not the receptor on their cell surface necessary for intracellular inhibition of ATIII.

Our strong *in vitro* efficacy was not transferred into *in vivo* studies. There might be several reasons for this result: (1.) Inhibition at entry level might have less efficacy because the virus has already infected the host cell in our *in vivo* system. (2.) The 60 kDa molecule might have difficulties to diffuse out of the gel polymer structure and to reach the target cells. Therefore, new delivery methods need to be tested to rule out this possibility. It might be necessary to couple the ATIII product with a small molecule antiviral agent to develop a combination product with multiple modalities of HSV-1 inhibition. This product might more effectively prevent virus transmission in both direct and indirect ways, will exhibit enhanced potency over the individual components of the combination product, and should be relatively insensitive to the selection of resistant virus strains.

V. CONCLUSION

Hep-ATIII belongs to a class of proteins termed serpins which have broad-spectrum antiviral activity. Our data show that hep-ATIII had a stronger inhibition capacity than ACV *in vitro*. Our results also indicate that inhibition was at the entry level and capable to inhibit

ACV-resistant HSV-1. Combining hep-ATIII with ACV might be one possibility to block the virus at two steps in its life-cycle which might slow down the occurrence of resistant virus typical for ACV long-term treatment of immunocompromised patients. Our data also indicated that novel delivery techniques need to be employed to increase *in vivo* efficacy.

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Table I

Scale for Lesions Symptoms

Scale	Symptoms
0.0	normal
0.5	whole face red & swollen, 1–2 discrete lesions on face
1.0	3–5 discrete lesions on face
1.5	25% confluency or scabs on face
2.0	50% confluency or scabs on face
2.5	75% confluency or scabs on face
3.0	75–100% confluency or scabs on face
3.5	2.5% necrotic tissue
4.0	50% necrotic tissue
4.5	75% necrotic tissue
5.0	75–100% necrotic tissue

Table II

Activity of Hep-ATIII in compare to ACV incytoprotection assays with different strains of viruses and target cells[1]

Compound (μM)	Vero HSV-1_{FL}		Vero HSV-1_{HF}		Vero HSV-1_{KOS}		HFF HSV-1_{FL}		HFF HSV-1_{KOS}		HFF HSV-$1_{MacIntyre}$	
	EC_{50}	Fold ACV activity	EC_{50}	Fold ACV activity	EC_{50}	Fold ACV activity	EC_{50}	Fold ACV activity	EC_{50}	Fold ACV activity	EC_{50}	Fold ACV activity
Hep-ATIII	2.3	3.2	2.2	2.7	1.4	1.8	2.8	3.1	1	10	6.3	0.6

[1] HFF cells (less than 10 passages) were added to a 24-well flat-bottom plate at a density of 1×10^5 cells/well 24 hours prior to assay initiation. Virus diluted to a pre-determined titer (20–30 pfu/well) was then added to the plates for 1 hour. Serially diluted compound was added to the cells in triplicate to the infected monolayers. The plates were incubated for 3 days at 37°C/5% CO_2. Efficacy was evaluated with a plaque quantification endpoint by staining the monolayers with crystal violet. Cytotoxicity was measured in parallel.

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Table III

Effect of topical treatment with heparin activated antithrombin III (hep-ATIII) on the mortality of SKH-1 mice infected orofacially with HSV-1

	Mortality				
Treatment[a]	**Number**	**Percent**	**P-value**	**MDD ± STD**[b]	**P-value**
Vehicle					
Carbopol gel	9/10	90	---	7.4 ± 1.7	---
Zovirax					
5% ACV	0/10	0	<0.001	---	---
Hep-ATIII					
10 mg/ml	4/10	40	<0.01	7.5 ± 0.6	NS[c]

[a] Topical treatments with vehicle, ACV, and hep-ATIII were applied topically 3 times daily for 7 consecutive days in 6 hour intervals beginning 24 hours post viral inoculation.

[b] MDD = Mean Day of Death. STD = Standard Deviation.

[c] NS = Not significant when compared to the appropriate vehicle control.

Table IV

Effect of topical treatment with heparin activated antithrombin III (hep-ATIII) on the facial lesion development of SKH-1 mice infected orofacially with HSV-1

Treatment[a]	#With Lesions/ #Inoculated	Lesion-Day AUC	P-Value	Mean Peak Lesion Score (AUC)	P-Value
Vehicle					
Carbopol gel	10/10	17.4	---	3.0 ± 0.2	---
Zovirax					
5 % ACV	10/10	4.1	NS[b]	1.3 ± 0.5	NS[b]
Hep-ATIII					
10 mg/ ml	10/10	12.4	NS[b]	2.9 ± 0.3	NS[b]

[a] Topical treatments with vehicle, ACV, and hep-ATIII were applied topically 3 times daily for 7 consecutive days in 6 hour intervals beginning 24 hours post viral inoculation.

[b] NS = non-significant when compared to the appropriate vehicle controls.

Table V

Virus titers in swabs of heparin activated antithrombin III (hep-ATIII) treated SKH-1 Mice

Treatment[a]	#Virus Positive/ Inoculated	Virus Titer-Day AUC	P-Value	Mean Peak Virus Score (AUC)	P-Value
Vehicle					
Carbopol gel	10/10	36.8	---	6.6 ± 0.4	---
Zovirax					
5 % ACV	10/10	17.2	<0.01	5.2 ± 0.8	<0.05
Hep-ATIII					
10 mg/ ml	10/10	29.5	0.09	5.8 ± 0.7	NS[b]

a. Topical treatments with vehicle, ACV, and hep-ATIII were applied topically 3 times daily for 7 consecutive days in 6 hour intervals beginning 24 hours post viral inoculation.

b. NS = Non-significant when compared to the appropriate vehicle controls.