



LUND UNIVERSITY

Isolation and characterization of a small antiretroviral molecule affecting HIV-1 capsid morphology

Abdurahman, Samir; Végvári, Ákos; Levi, Micheal; Höglund, Stefan; Högberg, Marita; Tong, Weimin; Romero, Ivan; Balzarini, Jan; Vahlne, Anders

Published in:
Retrovirology

DOI:
[10.1186/1742-4690-6-34](https://doi.org/10.1186/1742-4690-6-34)

2009

[Link to publication](#)

Citation for published version (APA):

Abdurahman, S., Végvári, Á., Levi, M., Höglund, S., Högberg, M., Tong, W., Romero, I., Balzarini, J., & Vahlne, A. (2009). Isolation and characterization of a small antiretroviral molecule affecting HIV-1 capsid morphology. *Retrovirology*, 6(34). <https://doi.org/10.1186/1742-4690-6-34>

Total number of authors:
9

General rights

Unless other specific re-use rights are stated the following general rights apply:
Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain
- You may freely distribute the URL identifying the publication in the public portal

Read more about Creative commons licenses: <https://creativecommons.org/licenses/>

Take down policy

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

LUND UNIVERSITY

PO Box 117
221 00 Lund
+46 46-222 00 00

This Provisional PDF corresponds to the article as it appeared upon acceptance. Fully formatted PDF and full text (HTML) versions will be made available soon.

Isolation and characterization of a small antiretroviral molecule affecting HIV-1 capsid morphology

Retrovirology 2009, **6**:34 doi:10.1186/1742-4690-6-34

Samir Abdurahman (Samir.abdurahman@ki.se)
Akos Vegvari (akos.vegvari@elmat.lth.se)
Michael Levi (Michael.Levi@tripep.se)
Stefan Hoglund (stefan.hoglund@biorg.uu.se)
Marita Hogberg (marita.hogberg@chemilia.com)
Weimin Tong (weimin.tong@chemilia.com)
Ivan Romero (ivan.romero@ki.se)
Jan Balzarini (jan.balzarini@rega.kuleuven.ac.be)
Anders Vahlne (anders.vahlne@ki.se)

ISSN 1742-4690

Article type Research

Submission date 22 December 2008

Acceptance date 8 April 2009

Publication date 8 April 2009

Article URL <http://www.retrovirology.com/content/6/1/34>

This peer-reviewed article was published immediately upon acceptance. It can be downloaded, printed and distributed freely for any purposes (see copyright notice below).

Articles in *Retrovirology* are listed in PubMed and archived at PubMed Central.

For information about publishing your research in *Retrovirology* or any BioMed Central journal, go to

<http://www.retrovirology.com/info/instructions/>

For information about other BioMed Central publications go to

<http://www.biomedcentral.com/>

© 2009 Abdurahman *et al.*, licensee BioMed Central Ltd.

This is an open access article distributed under the terms of the Creative Commons Attribution License (<http://creativecommons.org/licenses/by/2.0>), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Isolation and characterization of a small antiretroviral molecule affecting HIV-1 capsid morphology

Samir Abdurahman¹, Ákos Végvári³, Michael Levi², Stefan Höglund⁴, Marita Högberg⁵, Weimin Tong⁵, Ivan Romero⁵, Jan Balzarini⁶, and Anders Vahlne^{1§}

¹Division of Clinical Microbiology, Karolinska Institutet, F68 Karolinska University Hospital Huddinge, SE-141 86 Stockholm, Sweden

²Tripep AB, Hälsovägen 7, SE-141 57 Huddinge, Sweden

³Department of Electrical Measurements, Lund University, SE-221 00 Lund, Sweden

⁴Department of Biochemistry, Uppsala University, SE-751 23 Uppsala, Sweden

⁵Chemilia AB, SE-141 83 Huddinge, Sweden

⁶Rega Institute for Medical Research, Katholieke Universiteit Leuven, Minderbroedersstraat 10, B-3000 Leuven, Belgium

§ Corresponding author: Professor Anders Vahlne, M.D., Ph.D.

Email: anders.vahlne@ki.se Telephone no.: +46 8 5858 1313

Facsimile number: +46 8 5858 7931

Email addresses:

SA: Samir.abdurahman@ki.se

ÁV: akos.vegvari@elmat.lth.se

ML: Michael.Levi@tripep.se

SH: stefan.hoglund@biorg.uu.se

MH: marita.hogberg@chemilia.com

WT: weimin.tong@chemilia.com

IR: ivan.romero@ki.se

JB: jan.balzarini@rega.kuleuven.ac.be

AV: anders.vahlne@ki.se

Abstract

Background

Formation of an HIV-1 particle with a conical core structure is a prerequisite for the subsequent infectivity of the virus particle. We have previously described that glycineamide (G-NH₂) when added to the culture medium of infected cells induces non-infectious HIV-1 particles with aberrant core structures.

Results

Here we demonstrate that it is not G-NH₂ itself but a metabolite thereof that displays antiviral activity. We show that conversion of G-NH₂ to its antiviral metabolite is catalyzed by an enzyme present in bovine and porcine but surprisingly not in human serum. Structure determination by NMR suggested that the active G-NH₂ metabolite was α -hydroxy-glycineamide (α -HGA). Chemically synthesized α -HGA inhibited HIV-1 replication to the same degree as G-NH₂, unlike a number of other synthesized analogues of G-NH₂ which had no effect on HIV-1 replication. Comparisons by capillary electrophoresis and HPLC of the metabolite with the chemically synthesized α -HGA further confirmed that the antiviral G-NH₂-metabolite indeed was α -HGA.

Conclusions

α -HGA has an unusually simple structure and a novel mechanism of antiviral action. Thus, α -HGA could be a lead for new antiviral substances belonging to a new class of anti-HIV drugs, i.e. capsid assembly inhibitors.

Background

During or concomitant with the HIV-1 release from infected cells, the Gag precursor protein (p55) is cleaved sequentially into matrix (MA/p17), capsid (CA/p24), nucleocapsid (NC/p7) and p6. Thus, proteolytic cleavage of p55 within the budded particle triggers a morphological change of the core which transforms it from a spherical [1] to a conical core structure consisting of approximately 1,500 p24 molecules [2-4]. The conical core formation not only results as a consequence of the proteolytic cleavage of p55 but also from substantial conformational changes and rearrangements of the p24 [1] which is connected to one another through N-terminal hexamer and C-terminal dimer formations [5-8]. Acquisition of virion infectivity, reverse transcription, and subsequent dissociation of the capsid core are all critically dependent on just the right semi-stability of the capsid cone structure, which in turn is made up of multiple semi-stable non-covalent p24-p24 interactions [9]. Thus, proper structural rearrangement of p24 after Gag cleavage is a crucial step and is a highly conserved feature in most retroviruses [10]. This makes p24 of interest as a target for developing new antiviral drugs.

There are twenty-five approved drugs that belong to six different antiretroviral classes for the treatment of HIV-patients [11]. The majority of these drugs control HIV-1 infection by targeting the two viral enzymes reverse transcriptase and protease [12]. A 36 amino acid peptide binding to the transmembrane glycoprotein gp41 inhibiting the fusion of the viral envelope with the plasma membrane is also used [13, 14]. Two other classes of antiretroviral drugs, a CCR5 co-receptor antagonist entry inhibitor [15] and an integrase inhibitor [16, 17], have also recently been approved. Other drugs being developed include zinc finger inhibitors affecting the RNA binding of the nucleocapsid protein (NC, p7) [18, 19], and capsid maturation inhibitors [20-22].

We have previously shown that the tripeptide glycyl-prolyl-glycineamide (GPG-NH₂) cleaved to G-NH₂ by dipeptidyl peptidase CD26, present in both human and fetal calf serum, affects proper HIV-1 capsid assembly and infectivity [23-26]. Here we show that G-NH₂ by itself does not affect HIV-1 replication, but displays antiviral effect only when converted to a metabolite by a yet uncharacterized enzyme present in porcine or bovine serum but not in human serum. The metabolite was identified as the small molecule α -hydroxy-glycineamide (α -HGA) having a molecular mass of only 90 Daltons, a molecule which we recently showed could inhibit HIV-1 replication [27].

Results

The effect of serum on the antiviral activity of glycineamide (G-NH₂)

The antiviral activity of G-NH₂ was tested in HIV-1 infected H9 cells cultured in medium containing human (HS), porcine (PS) or fetal calf serum (FCS). When FCS was used, 100 μ M G-NH₂ repeatedly abolished HIV infectivity (Fig. 1A, FCS). Similar results were also obtained when infected cells were cultured in PS (data not shown). However, no antiviral activity was observed when the infected cells were cultured in HS (Fig. 1A, HS). An explanation for this could be that G-NH₂ had to be converted by an enzyme present in FCS and PS, but not in HS, to acquire its antiviral activity. To test this hypothesis, 1 mM G-NH₂ was dialyzed against FCS (pre-dialyzed against PBS five times to clear it from low molecular weight material) at 37°C over night. The dialysis solution (DS) obtained which contained the presumed G-NH₂ metabolite, was then added to infected H9 cells cultured in medium containing HS (Fig. 1B, DS). Infected cell cultures to which 100 μ M G-NH₂ or no drug had been added served as controls. The results of a typical experiment are shown in Fig. 1B. G-NH₂ showed no antiviral activity, however, infected cells cultured in human serum with DS at

a 1/10 dilution, corresponding to ~100 μM of possible G-NH₂-FCS product, showed virus replication that was completely inhibited (Fig. 1B, DS).

To further test if G-NH₂ was converted to the active antiviral substance by an enzyme present in porcine or calf serum, HIV-1-infected H9 cells were cultured in medium containing normal PS or boiled PS (BPS). The cells were then treated with 100 μM G-NH₂, with the DS at a 1/10 dilution or were left untreated. A typical experiment is depicted in Fig. 1C. Infected cells without any test compound and cultured in medium containing PS or BPS served as controls (Fig. 1C, Untreated). In contrast to what was observed in cells cultured with BPS and treated with DS, G-NH₂ showed no antiviral activity in cells cultured with medium containing BPS (Fig. 1C). DS, however, repeatedly inhibited HIV replication regardless of the infected cells being cultured in the presence of PS or BPS (Fig. 1C, DS).

HPLC analysis of the unknown metabolite of G-NH₂

DS derived from 1 mM G-NH₂ dialyzed against pre-washed HS or PS was analyzed by HPLC using a cationic ion-exchange column. With G-NH₂ dialyzed against PS at 37°C (Fig. 2A) but not at 4°C (Fig. 2B), a peak designated Met-X (retention time at 2.9 min) was always observed in addition to the G-NH₂ peak (at 6.2 min). Dialysis of G-NH₂ in HS gave no such change in the HPLC pattern (Fig. 2C). The unknown peak fraction obtained by dialysis of G-NH₂ at 37°C was also isolated and tested for its antiviral activity (see below).

Furthermore, we tested a number of different animal sera for their ability to convert ¹⁴C-G-NH₂ to the antiviral metabolite X (Met-X). The conversion of G-NH₂ to Met-X was detected by the migration pattern in HPLC. As shown in Figure 3, sera from human, rat, mouse, and bird did not convert G-NH₂ but sera from rabbit, monkey, cat, dog, pig, horse and cow were successful in conversion.

Identification of metabolite-X (Met-X) by NMR

$^{13}\text{C}_2/^{15}\text{N}$ -labeled glycine (Fig. 4A) was transformed to $^{13}\text{C}_2/^{15}\text{N}$ -labeled G-NH₂ (Fig. 4B) by Fmoc peptide synthesis. In order to produce $^{13}\text{C}_2/^{15}\text{N}$ -labeled Met-X (Fig. 4C), $^{13}\text{C}_2/^{15}\text{N}$ -labeled G-NH₂ (Fig. 4B) was dialyzed against PS or FCS as described above. The $^{13}\text{C}^{15}\text{N}$ -labeled Met-X was then purified by HPLC and the peak fraction containing labeled Met-X was concentrated by lyophilization before being analyzed by NMR.

Based on the NMR analysis (^1H NMR, coupled ^1H - ^{13}C NMR, and 2D ^1H - ^{15}N HSQC NMR) one of the possible structures of the unknown compound Met-X was determined as α -hydroxy-glycineamide (α -HGA).

Comparison of Met-X with α -HGA

α -HGA was chemically synthesized and compared to Met-X. The HPLC chromatogram of α -HGA was identical to that of Met-X (Fig. 5A). Furthermore, the antiviral activity of the HPLC peak fraction identified as Met-X and α -HGA were tested in H9 cells infected with the HIV-1 SF-2 virus and in chronically infected ACH-2 cells. Both substances had similar antiviral activity (data not shown). The HPLC peak fractions identified as Met-X and α -HGA were also analyzed by capillary electrophoresis using bare silica capillaries under the same experimental conditions. The analysis of Met-X revealed some impurities which were well separated from the major peak containing Met-X (Fig. 5B). The pure α -HGA sample gave a symmetrical single peak, which had strikingly similar migration times to Met-X. The reproducibility was high (RSD=1.14%; n=4). Comparison of the UV spectra of the substances in separate experiments further revealed identical absorbance properties. Furthermore, the structural information gained by proton and carbon NMR analyses resulted in identical chemical shift values (data not shown).

Both α -HGA and Met-X treatment at concentrations corresponding to 10 μ M resulted in similarly significant changes in virion core morphology (data not shown). Pleomorphic virus particles with distorted, irregular packing of aberrant core structures, partly devoid of dense core material, were seen. Virions having double core structures and occasionally viral cores bulging off from viral envelope were also observed.

Anti-HIV activities of α -HGA and other related test compounds

α -HGA and some other structurally related compounds (Fig. 6A) at drug concentrations of 100 μ M were tested for a possible inhibitory effect on HIV-1 replication in infected H9 cells in the presence of FCS. As shown in Fig. 6B, both α -HGA and G-NH₂ abolished HIV-1 replication. By contrast, oxamic acid, oxamide, α -methoxy glycineamide, and Boc- α -methoxy glycineamide did not show any significant effect on HIV-1 replication. The 50% inhibitory concentration (IC₅₀) in HIV-1 SF-2 infected H9 cells ranged from 4 to 6 μ M for both α -HGA and Met-X. A typical dose response curve for α -HGA is depicted in Fig. 6C.

Discussion

In this study, we were able to identify, isolate and characterize a novel antiretroviral glycineamide (G-NH₂)-derived metabolite (Met-X) obtained after incubation of G-NH₂ in porcine (PS) or fetal calf (FCS) serum. Dialysis of G-NH₂ against FCS at 4°C or boiled PS gave no Met-X, indicating that the enzyme responsible for converting G-NH₂ to Met-X is temperature-dependent and heat-sensitive. Furthermore, unlike in FCS or in PS, G-NH₂ could not be converted to Met-X when incubated in human serum at 37°C, suggesting that humans lack either the active enzyme or a necessary co-factor. Interestingly, humans seem to share

this inability to convert G-NH₂ with mice, rats and birds. However, other species such as non-human primates can readily convert G-NH₂ to Met-X.

Here we characterized Met-X by NMR and this unknown compound was identified as α -hydroxy-glycineamide (α -HGA). In addition, with NMR, HPLC and capillary electrophoresis analysis of Met-X and the synthesized α -hydroxy-glycineamide the same chemical structure was determined. Therefore, it is very likely that these two compounds are identical chemical entities. The antiviral activity of the Met-X purified by cation exchange chromatography and identified as α -HGA by NMR was also confirmed both in H9 cells infected with the HIV-1 SF-2 virus and chronically infected ACH-2 cells. Consistent with previous reports on GPG-NH₂ and G-NH₂, the addition of Met-X or α -HGA to the culture medium of infected cells resulted in HIV-1 particles with aberrant core morphology.

The reduction in infectivity was not due to cytotoxicity, since neither Met-X nor α -HGA at concentrations up to 1,000 μ M has any effect on the cell viability of PBMC or a number of other cell lines [27]. Furthermore, α -HGA had no mitogenic activity against human PBMCs at concentrations of up to 2,000 μ M.

Two other compounds that inhibit or interfere with the HIV-1 capsid (p24/CA) maturation or assembly have previously been reported [20, 21, 28]. PA-457 [20, 22], is a compound that binds to the proteolytic cleavage site of the p24 precursor (p25/CA-SP1) and thereby affects its maturation to p24. α -HGA does not affect the proteolytic processing of p25 [27]. The other compound reported by Tang *et al.* describes the binding of *N*-(3-chloro-4-methylphenyl)-*N'*-2-(5-[dimethylamino-methyl]-2-furyl)-methylsulfanyl-ethyl urea (CAP-1) to the N-terminal domain of p24 [21]. CAP-1 affects HIV-1 capsid cone formation but did not prevent virus

release [21]. However, α -HGA, which is comparatively a small molecule, specifically affected HIV-1 CA assembly and cone formation, possibly by binding to the hinge region between the N- and C-terminal domains of p24 [27]. A 12-mer alpha-helical peptide (CAI) was also shown to interfere with p24 dimerization, but not with HIV-1 replication in cell culture due to the lack of cell penetration [28, 29]. However, more recently a structure-based rational design was used to stabilize the alpha-helical structure of CAI and convert it to a cell-penetrating peptide (CPP) displaying antiviral activity [30].

Conclusions

In this study, we have reported that G-NH₂ by itself has no anti-viral activity but is converted to a small (molecular mass 90) anti-retroviral compound when incubated in some animal sera. The new compound was identified as α -HGA, which has an unusually simple structure and a novel mechanism of antiviral action. Thus, α -HGA could be a lead for new antiviral substances belonging to a new class of anti-HIV drugs, i.e. capsid assembly/maturation inhibitors.

Methods

Cells, media and reagents

Peripheral blood mononuclear cells (PBMC), H9 and ACH-2 cells were cultured in complete RPMI-1640, and HeLa-tat cells was cultured in complete DMEM medium supplemented with 10% serum and antibiotics. Porcine and human sera (PS and HS) (Biomeda), fetal calf serum (FCS; Invitrogen) oxamic acid and oxamide (Sigma) were used. Glycineamide (G-NH₂) and α -hydroxy glycineamide (α -HGA; manufactured to order by Pharmatory Oy, Oulu, Finland) were kindly provided by Tripep AB, Stockholm, Sweden. ¹³C₂/¹⁵N-labeled Fmoc-glycine (Isotech) was transformed to ¹³C₂/¹⁵N-labeled G-NH₂ by Fmoc peptide synthesis. ¹³C₂/¹⁵N-

labeled G-NH₂ was dialyzed against PS or FCS to produce ¹³C₂/¹⁵N-labeled metabolite of G-NH₂ which will be referred to as Met-X (Fig. 22A, B and C).

Inhibition of viral infectivity

HIV-1 stock of SF-2 from H9 cells was prepared as described previously [31], and 50% tissue culture infectious dose (TCID₅₀) was determined. H9 cells were infected with SF-2 at 100 TCID₅₀ by incubating for 2 hours at 37°C. The cells were then pelleted, washed and resuspended in complete RPMI medium containing HS or PS, and the test compound was added. Cells were cultured for 11 days, and the growth medium was changed seven days post-infection. The HIV-1 p24 antigen contents were assayed at day 7 and 11 post infection essentially as described elsewhere [32] (see below). For RT-assay, the manufacturer's procedure was followed (Cavidi Tech AB, Uppsala, Sweden).

HPLC analysis and purification of Met-X

¹³C₂/¹⁵N-labeled or unlabeled G-NH₂ was enzymatically transformed to Met-X by dialysis against FCS or PS at 37°C. Dialysis was performed with 10 ml of serum in a dialysis tubing (5 kD MWCO) that was prewashed by dialyzing 5× against PBS under constant stirring. After 24 hours, the dialysis solution (DS) containing Met-X was analyzed by injecting it onto a 250 × 10 mm, 5 μm cationic ion-exchange column, Theoquest Hypersil SCX, (Thermo), with 90% 0.1 M KH₂PO₄ pH 4.5/10% acetonitrile as mobile phase at isocratic flow. The absorbance was measured at 206 nm. Lyophilized ¹³C₂/¹⁵N-labeled Met-X was also analyzed as above except that a mobile phase of 90% 2.5 mM formic acid pH 3 / 10% acetonitrile was used. All HPLC chromatograms were compared using retention time as an indicator. Once the structure of Met-X was indicated by NMR (see below) to be α-HGA, the HPLC properties of Met-X and chemically synthesized α-HGA were analyzed under the same conditions.

Compound characterization by NMR spectroscopy

The HPLC peak fraction containing $^{13}\text{C}_2/^{15}\text{N}$ -labeled Met-X was isolated, lyophilized, and analyzed with NMR. Due to the low natural abundance of ^{13}C - and ^{15}N -nuclei, a commercially available labeled glycine with two 99% ^{13}C - and one 99% ^{15}N -isotopes (Fig. 4A) was used as starting material. The labeled glycine was transformed into G-NH₂ (Fig. 4B) which was dialyzed against PS or FCS to obtain labeled Met-X. The $^{13}\text{C}/^{15}\text{N}$ -labeled Met-X was purified by HPLC and concentrated by lyophilization before being analyzed with NMR. The samples were analyzed on a Bruker DPX 300 MHz, a Jeol Eclipse⁺500 MHz and Bruker DMX 600 MHz spectrometers.

Comparison of Met-X with α -HGA by capillary electrophoresis

Capillary electrophoresis experiments were carried out at 20°C with a BioFocus 3000 system (Bio-Rad) which was equipped with a fast scanning UV-Vis detector. Fused silica tubing (50 and 365 μm inner and outer diameter, respectively) was purchased from MicroQuartz and cut to a length of 23 cm (with 18.5 cm effective length). Sodium phosphate buffer (0.05 M) at pH 7.4 was used as a background electrolyte. The polarity was set from positive to negative (with the detection point closer to the cathode). The capillary was flushed with the buffer for 1 minute before each run. The Met-X solution obtained from the dialysis procedure was diluted two fold in the buffer solution and filtered through a syringe disc filter (Ultra free-MC 5 000 NMWL, Millipore) prior to injection by pressure (3 psi·s). α -HGA was dissolved in the buffer at 10 mM concentration and injected by pressure (3 psi·s). The applied voltage was 10 kV in all experiments resulting in 50 μA current.

ELISA

p24-ELISA of infected cell culture supernatants was performed essentially as described elsewhere [32]. Briefly, rabbit anti-p24 coated micro-well plates (MWP) were blocked with 3% BSA in PBS at 37°C for 30 minutes. Supernatants from infected cells were added to the plates, followed by incubated at 37°C for 1 hour. The MWPs were washed three times, and biotinylated anti-p24 antibody (1:1 500) was added. One hour after incubation, the MWPs were washed and incubated with HRP-conjugated streptavidine (1:2 000) for 30 minutes. Finally, the MWPs were washed and detected by adding the substrate o-Phenylenediamine Dihydrochloride (Sigma). Recombinant p24 at fixed concentrations was used as a standard. The plates were read in a Labsystems multiscan MS spectrometer. For RT-ELISA, the manufacturer's procedure was followed (Cavidi Tech).

Anti-HIV activities of α -HGA and other related test compounds

The antiviral activity of α -HGA and some other structurally related compounds was tested in infected H9 cells in the presence of FCS at drug concentrations of 100 μ M. H9 cells were infected as described above and cultured in medium containing oxamic acid, oxamide, α -methoxy glycineamide and Boc- α -methoxy glycineamide.

Conversion of G-NH₂ to α -HGA by different sera

The sera from different animal species were diluted 10-fold in 50 mM potassium phosphate buffer pH 8.0. To 100 μ l (10% serum) were added 0.1 μ Ci [¹⁴C]G-NH₂ (radiospecificity: 56 mCi/mmol), and the samples were incubated for 1, 6 or 24 hours at 37°C. At these time points, 200 μ l cold methanol was added, and the samples were left on ice for another 15 minutes. After centrifugation at 15,000 rpm, the supernatants were subjected to HPLC analysis using a SCX-partisphere column (Whatman). The following gradient was used to

separate G-NH₂ and Met-X (α -HGA): 5 mM buffer B (5 mM NH₄H₂PO₄ pH 3.5) (10 minutes); linear gradient to 83% buffer C (0.3 M NH₄H₂PO₄ pH 3.5) (6 minutes); equilibration 83% buffer C (2 minutes); linear gradient to 100% buffer B (6 minutes); equilibration 100% buffer B (6 minutes). The retention times of G-NH₂ and α -HGA were 12 and 2 minutes, respectively.

Transmission electron microscopy (TEM)

H9 cells were infected with HIV-1 SF-2 at 100 TCID₅₀ by incubating for 2 hours at 37°C.

After seven days of incubation, medium containing Met-X or α -HGA was added. Cells were cultured for an additional four days, and progeny virus was analyzed by transmission electron microscopy (TEM). The HIV-1-infected H9 cells were fixed freshly upon embedding in epon, essentially as described before [24]. Sections were made approximately 60 nm thick to allow accommodation of the volume of the core structure parallel to the section plane. Duplicate samples were used and minimal beam dose technique was employed throughout. Evaluation of morphology was done with series of electron micrographs to depict different categories of virus morphology. Similar results were also obtained with chronically infected ACH-2 cells induced to replicate HIV-1.

Competing interests

Author AV is a shareholder and a director of the board of Tripep AB, and author ML is an employee of Tripep AB.

Authors' contributions

SH performed electron microscopy, ÁV capillary electrophoresis, and ML HPLC analysis.

MH together with WT and IR performed NMR analysis. JB performed the experiments with different animal sera. SA performed all other experiments in the study and wrote the manuscript with AV. AV is the principal investigator and conceived of the study. All authors read and approved the manuscript.

Acknowledgements

We thank Pia Österwall and Sung Oun Stenberg for help with the dialysis and antiviral assay.

We thank the original donors of the following reagents that were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: the CD4⁺ cell lines H9 [33] and ACH-2 [34], and the CD4⁻ cell line HeLa-tat-III [35]. This work was supported by grants from the K.U.Leuven (GOA-05/19), Swedish Research Council (grant no. K2000-06X-09501-10B), Swedish International development Cooperation Agency, SIDA (grant no. HIV-2006-050) and Tripep AB.

References

1. Briggs JA, Simon MN, Gross I, Krausslich HG, Fuller SD, Vogt VM, Johnson MC: **The stoichiometry of Gag protein in HIV-1.** *Nat Struct Mol Biol* 2004, **11**:672-675.
2. Lanman J, Lam TT, Barnes S, Sakalian M, Emmett MR, Marshall AG, Prevelige PE, Jr.: **Identification of novel interactions in HIV-1 capsid protein assembly by high-resolution mass spectrometry.** *J Mol Biol* 2003, **325**:759-772.
3. Li S, Hill CP, Sundquist WI, Finch JT: **Image reconstructions of helical assemblies of the HIV-1 CA protein.** *Nature* 2000, **407**:409-413.

4. Chertova E, Chertov O, Coren LV, Roser JD, Trubey CM, Bess JW, Jr., Sowder RC, 2nd, Barsov E, Hood BL, Fisher RJ, et al: **Proteomic and biochemical analysis of purified human immunodeficiency virus type 1 produced from infected monocyte-derived macrophages.** *J Virol* 2006, **80**:9039-9052.
5. Gamble TR, Vajdos FF, Yoo S, Worthylake DK, Houseweart M, Sundquist WI, Hill CP: **Crystal structure of human cyclophilin A bound to the amino-terminal domain of HIV-1 capsid.** *Cell* 1996, **87**:1285-1294.
6. Momany C, Kovari LC, Prongay AJ, Keller W, Gitti RK, Lee BM, Gorbalenya AE, Tong L, McClure J, Ehrlich LS, et al: **Crystal structure of dimeric HIV-1 capsid protein.** *Nat Struct Biol* 1996, **3**:763-770.
7. Berthet-Colominas C, Monaco S, Novelli A, Sibai G, Mallet F, Cusack S: **Head-to-tail dimers and interdomain flexibility revealed by the crystal structure of HIV-1 capsid protein (p24) complexed with a monoclonal antibody Fab.** *Embo J* 1999, **18**:1124-1136.
8. Gamble TR, Yoo S, Vajdos FF, von Schwedler UK, Worthylake DK, Wang H, McCutcheon JP, Sundquist WI, Hill CP: **Structure of the carboxyl-terminal dimerization domain of the HIV-1 capsid protein.** *Science* 1997, **278**:849-853.
9. Forshey BM, von Schwedler U, Sundquist WI, Aiken C: **Formation of a human immunodeficiency virus type 1 core of optimal stability is crucial for viral replication.** *J Virol* 2002, **76**:5667-5677.
10. Mortuza GB, Haire LF, Stevens A, Smerdon SJ, Stoye JP, Taylor IA: **High-resolution structure of a retroviral capsid hexameric amino-terminal domain.** *Nature* 2004, **431**:481-485.
11. De Clercq E: **The design of drugs for HIV and HCV.** *Nat Rev Drug Discov* 2007, **6**:1001-1018.

12. De Clercq E: **Emerging anti-HIV drugs.** *Expert Opin Emerg Drugs* 2005, **10**:241-273.
13. Root MJ, Steger HK: **HIV-1 gp41 as a target for viral entry inhibition.** *Curr Pharm Des* 2004, **10**:1805-1825.
14. Yu H, Tudor D, Alfsen A, Labrosse B, Clavel F, Bomsel M: **Peptide P5 (residues 628-683), comprising the entire membrane proximal region of HIV-1 gp41 and its calcium-binding site, is a potent inhibitor of HIV-1 infection.** *Retrovirology* 2008, **5**:93.
15. Groeschel HM: **Novel HIV treatment approved.** *Am J Health Syst Pharm* 2007, **64**:1886.
16. Traynor K: **Integrase inhibitor gains FDA approval.** vol. 64. pp. 2310-; 2007:2310-.
17. Anker M, Corales RB: **Raltegravir (MK-0518): a novel integrase inhibitor for the treatment of HIV infection.** vol. 17. pp. 97-103; 2008:97-103.
18. Darlix JL, Garrido JL, Morellet N, Mely Y, de Rocquigny H: **Properties, functions, and drug targeting of the multifunctional nucleocapsid protein of the human immunodeficiency virus.** *Adv Pharmacol* 2007, **55**:299-346.
19. Musah RA: **The HIV-1 nucleocapsid zinc finger protein as a target of antiretroviral therapy.** *Curr Top Med Chem* 2004, **4**:1605-1622.
20. Li F, Goila-Gaur R, Salzwedel K, Kilgore NR, Reddick M, Matallana C, Castillo A, Zoumplis D, Martin DE, Orenstein JM, et al: **PA-457: a potent HIV inhibitor that disrupts core condensation by targeting a late step in Gag processing.** *Proc Natl Acad Sci U S A* 2003, **100**:13555-13560.
21. Tang C, Loeliger E, Kinde I, Kyere S, Mayo K, Barklis E, Sun Y, Huang M, Summers MF: **Antiviral inhibition of the HIV-1 capsid protein.** *J Mol Biol* 2003, **327**:1013-1020.

22. Smith PF, Ogundele A, Forrest A, Wilton J, Salzwedel K, Doto J, Allaway GP, Martin DE: **Phase I and II study of the safety, virologic effect, and pharmacokinetics/pharmacodynamics of single-dose 3-o-(3',3'-dimethylsuccinyl)betulinic acid (bevrimat) against human immunodeficiency virus infection.** *Antimicrob Agents Chemother* 2007, **51**:3574-3581.
23. Su J, Andersson E, Horal P, Naghavi MH, Palm A, Wu YP, Eriksson K, Jansson M, Wigzell H, Svennerholm B, Vahlne A: **The nontoxic tripeptide glycyl-prolyl-glycine amide inhibits the replication of human immunodeficiency virus type 1.** *J Hum Virol* 2001, **4**:1-7.
24. Hoglund S, Su J, Reneby SS, Vegvari A, Hjerten S, Sintorn IM, Foster H, Wu YP, Nystrom I, Vahlne A: **Tripeptide interference with human immunodeficiency virus type 1 morphogenesis.** *Antimicrob Agents Chemother* 2002, **46**:3597-3605.
25. Balzarini J, Andersson E, Schols D, Proost P, Van Damme J, Svennerholm B, Horal P, Vahlne A: **Obligatory involvement of CD26/dipeptidyl peptidase IV in the activation of the antiretroviral tripeptide glycylprolylglycinamide (GPG-NH(2)).** *Int J Biochem Cell Biol* 2004, **36**:1848-1859.
26. Andersson E, Horal P, Jejcic A, Hoglund S, Balzarini J, Vahlne A, Svennerholm B: **Glycine-amide is an active metabolite of the antiretroviral tripeptide glycyl-prolyl-glycine-amide.** *Antimicrob Agents Chemother* 2005, **49**:40-44.
27. Abdurahman S, Vegvari A, Youssefi M, Levi M, Hoglund S, Andersson E, Horal P, Svennerholm B, Balzarini J, Vahlne A: **Activity of the small modified amino acid alpha-hydroxy glycineamide on in vitro and in vivo human immunodeficiency virus type 1 capsid assembly and infectivity.** *Antimicrob Agents Chemother* 2008, **52**:3737-3744.

28. Sticht J, Humbert M, Findlow S, Bodem J, Muller B, Dietrich U, Werner J, Krausslich HG: **A peptide inhibitor of HIV-1 assembly in vitro.** *Nat Struct Mol Biol* 2005, **12**:671-677.
29. Ternois F, Sticht J, Duquerroy S, Krausslich HG, Rey FA: **The HIV-1 capsid protein C-terminal domain in complex with a virus assembly inhibitor.** *Nat Struct Mol Biol* 2005, **12**:678-682.
30. Zhang H, Zhao Q, Bhattacharya S, Waheed AA, Tong X, Hong A, Heck S, Curreli F, Goger M, Cowburn D, et al: **A cell-penetrating helical peptide as a potential HIV-1 inhibitor.** *J Mol Biol* 2008, **378**:565-580.
31. Vahlne A, Horal P, Eriksson K, Jeansson S, Rymo L, Hedstrom KG, Czerkinsky C, Holmgren J, Svennerholm B: **Immunizations of monkeys with synthetic peptides disclose conserved areas on gp120 of human immunodeficiency virus type 1 associated with cross-neutralizing antibodies and T-cell recognition.** *Proc Natl Acad Sci U S A* 1991, **88**:10744-10748.
32. Horal P, Hall WW, Svennerholm B, Lycke J, Jeansson S, Rymo L, Kaplan MH, Vahlne A: **Identification of type-specific linear epitopes in the glycoproteins gp46 and gp21 of human T-cell leukemia viruses type I and type II using synthetic peptides.** *Proc Natl Acad Sci U S A* 1991, **88**:5754-5758.
33. Popovic M, Sarngadharan MG, Read E, Gallo RC: **Detection, isolation, and continuous production of cytopathic retroviruses (HTLV-III) from patients with AIDS and pre-AIDS.** *Science* 1984, **224**:497-500.
34. Clouse KA, Powell D, Washington I, Poli G, Strebel K, Farrar W, Barstad P, Kovacs J, Fauci AS, Folks TM: **Monokine regulation of human immunodeficiency virus-1 expression in a chronically infected human T cell clone.** *J Immunol* 1989, **142**:431-438.

35. Rosen CA, Haseltine WA, Lenz J, Ruprecht R, Cloyd MW: **Tissue selectivity of murine leukemia virus infection is determined by long terminal repeat sequences.** *J Virol* 1985, **55**:862-866.

Figure legends

Figure 1 - Antiviral activity of G-NH₂ and characterization of G-NH₂ metabolite obtained after dialysis against FCS or PS

(A) H9 cells (10^5) were infected with the SF-2 strain of HIV-1 and cultured in medium containing 100 μ M G-NH₂ and either 10% fetal calf serum (FCS) or human serum (HS). Ten days post-infection, the level of p24-antigen in the culture supernatants was assayed with a p24-ELISA. (B) H9 cells infected with the SF-2 strain of HIV-1 were cultured in medium containing 10% human serum (HS) and either 100 μ M G-NH₂ or a dialysis solution (DS) of 1/10 dilution of 1 mM G-NH₂ dialyzed against FCS. Untreated cultures without any addition of G-NH₂ or DS served as controls. (C) Infected H9 cells were cultured in the presence of 10% boiled porcine serum (BPS) or non-boiled porcine serum (PS). The infected cultures were cultured with the addition of 100 μ M G-NH₂, DS or were left untreated. Virus production was assessed using an RT assay. Error bars indicate standard deviations from quadruple cultures.

Figure 2 - HPLC analysis of G-NH₂ and the G-NH₂-derived metabolite Met-X

HPLC chromatograms of dialysis solution (DS) after dialyzing 1 mM G-NH₂ against porcine serum (PS) at 37°C (A) and at 4°C (B) or human serum at 37°C. The dialysis solutions were analyzed with a cationic ion-exchange column (Theoquest Hypersil SCX, Thermo), and the absorbance was measured at 206 nm.

Figure 3 - Conversion of G-NH₂ to Met-X in different sera

¹⁴C-labeled G-NH₂ was incubated with sera from different species at different time points as indicated in the figure. Conversion to Met-X was analyzed by HPLC. Percent conversion to Met-X for respective sera is depicted.

Figure 4 - Chemical structure and production of G-NH₂-derived metabolite after dialysis against porcine serum

The chemical structures of doubly labeled glycine with two ¹³C- and one ¹⁵N-isotopes (A) which was transformed into labeled glycineamide (B). The latter was dialyzed against porcine serum at 37°C, and the ¹³C₂¹⁵N-labeled product (C) here is referred to as Met-X. This compound was purified by HPLC and concentrated before being analyzed with NMR.

Figure 5 - Comparison of Met-X with α-HGA

HPLC analysis of synthetically produced α-HGA and Met-X, the latter produced enzymatically after dialyzing 1 mM G-NH₂ against PS at 37°C, is depicted in panel A, and capillary electrophoresis analysis of α-HGA and Met-X in panel B.

Figure 6 - Biological and antiviral comparison of α-HGA and some structurally related compounds

Chemical structures of glycine, glycineamide (G-NH₂), α-HGA, oxamic acid, oxamide, α-methoxy glycineamide and Boc-α-methoxy glycineamide (A). Antiviral activity of 100 μM of respective compound added to HIV-1 SF-2 infected H9 cells cultured in the presence of 10% fetal bovine serum (B). Dose response of the antiviral activity of synthetically produced α-HGA (C).

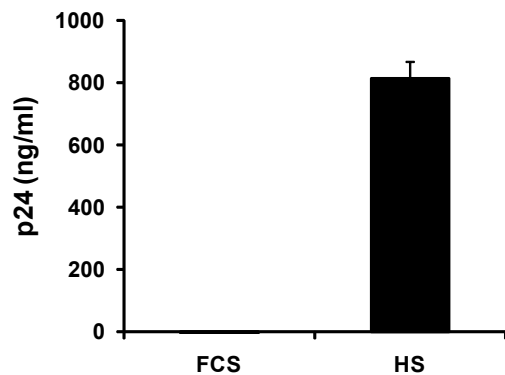
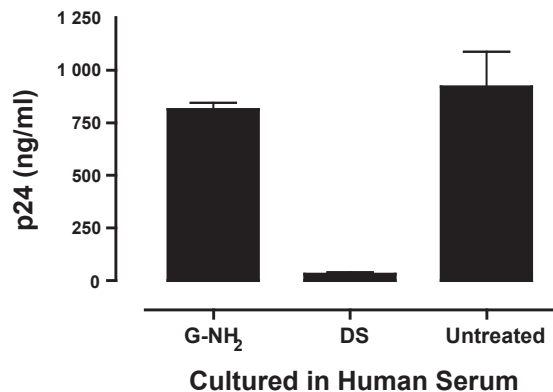
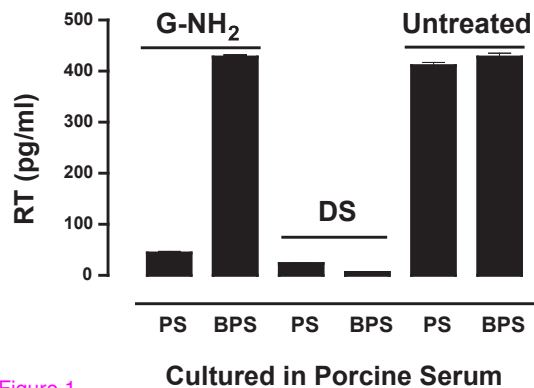
A**B****C**

Figure 1

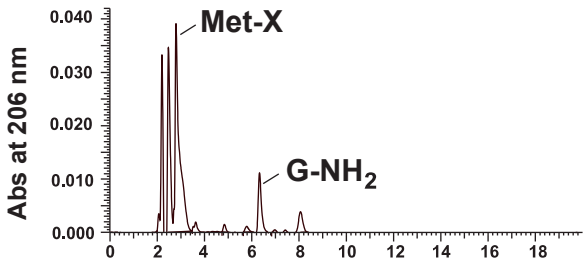
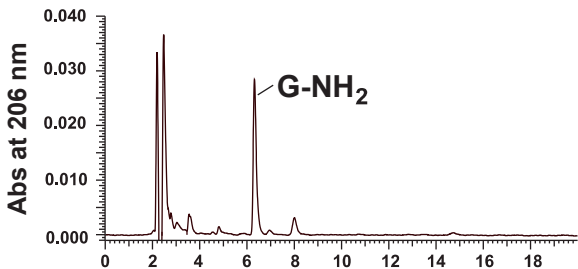
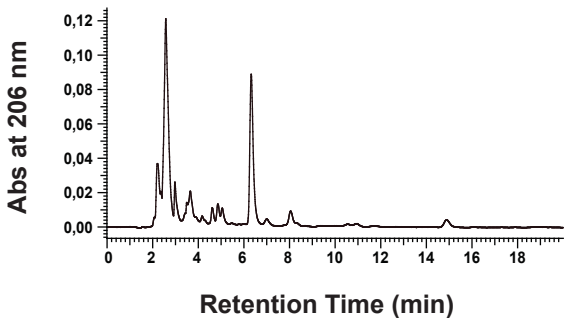
A**B****C**

Figure 2

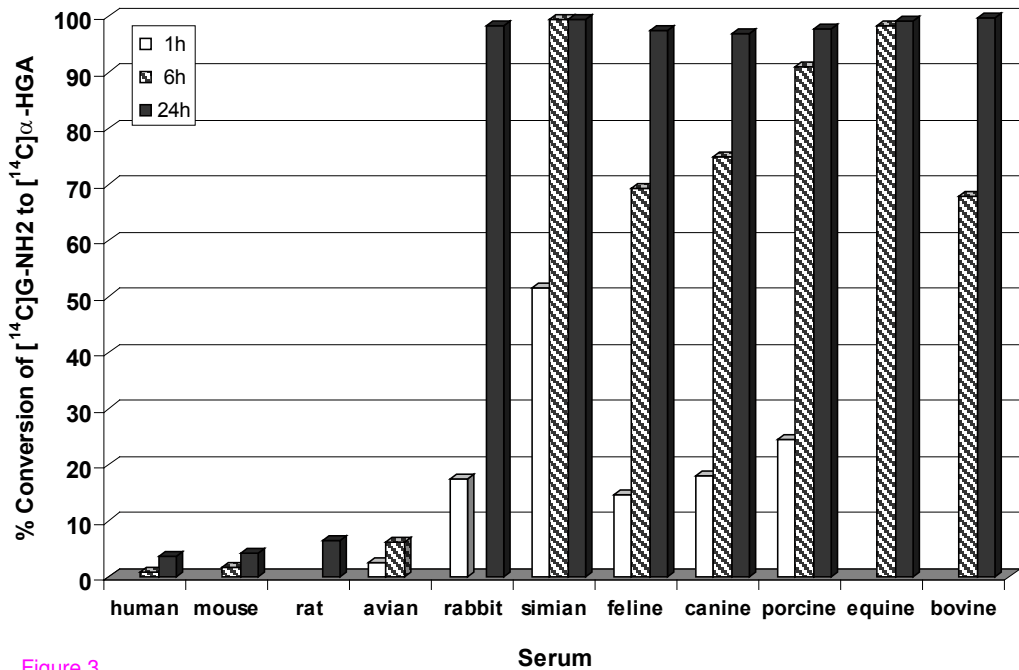


Figure 3

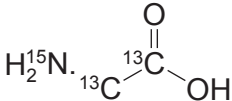
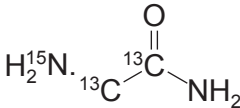
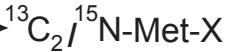
A**Glycine****B****Glycineamide****C****PS/FCS****Dialysis**

Figure 4

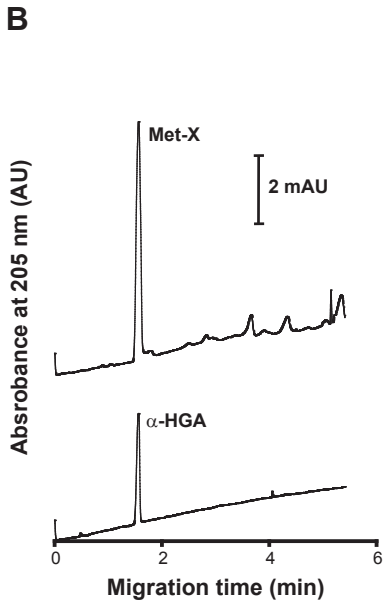
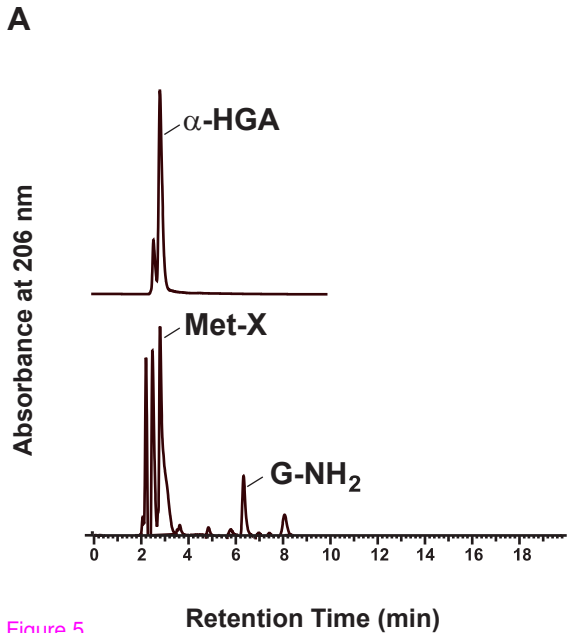


Figure 5

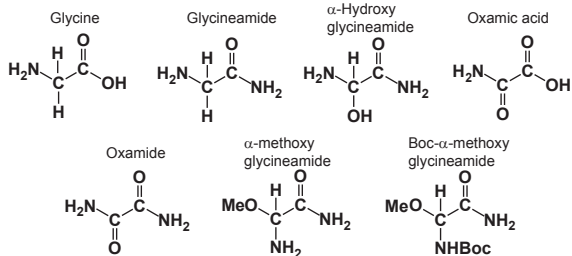
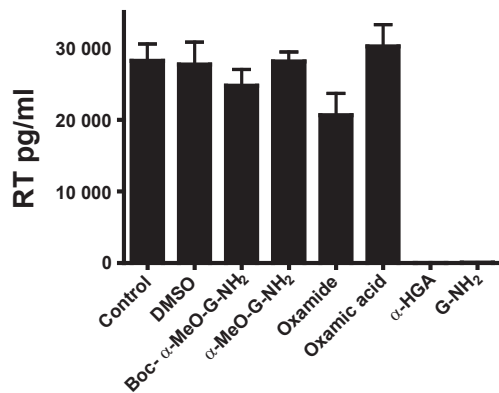
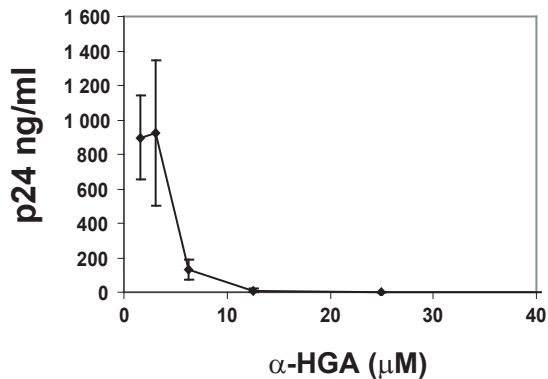
A**B****C**

Figure 6