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No cross-resistance or selection of HIV-1 resistant mutants in vitro to the antiretroviral tripeptide glycyl-prolyl-glycine-amide

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Abstract

The chemically modified tripeptide glycyl-prolyl-glycine-amide (GPG-NH_2) inhibits replication of HIV-1 in vitro, probably by interfering with capsid formation. This study was aimed at determining cross-resistance between antiretroviral drugs and GPG-NH_2, and whether resistance to GPG-NH_2 can be induced in vitro. Fifty-five clinical HIV-1 isolates with different resistance-related mutations were tested for susceptibility to GPG-NH_2. No correlation between NRTI-, NNRTI- or PI-resistance and efficacy of GPG-NH_2 was found, indicating the lack of cross-resistance. Serial passages were performed with GPG-NH_2, and with lamivudine, and genotypic or phenotypic changes were determined. Resistance to lamivudine was detected after six passages. No resistance to GPG-NH_2 was generated after 30 passages in two parallel series. However, one mutation (T107I) in the p24 gene was detected in both series, but this mutation was not associated with decreased sensitivity to GPG-NH_2.

Keywords: Glycyl-prolyl-glycine-amide; Tripeptides; Antiretroviral resistance

1. Introduction

Today, four classes of antiretroviral drugs are available for the treatment of HIV; nucleoside and nucleotide reverse transcriptase inhibitors (NRTIs, NtRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs), and protease inhibitors (PIs). In addition, a synthetic peptide that inhibits the fusion of HIV and the target cell has recently entered the market (LaBranche et al., 2001; Lawless et al., 1996). Drugs with similar or different modes of action are usually combined (HAART, highly active antiretroviral therapy) and thus suppress development of antiviral drug resistance (Balzarini, 1999; Carpenter et al., 2000; Collier et al., 1996). Development of resistance, severe side effects and poor compliance are major problems with HAART. In addition, HAART is not able to eradicate the virus, it only suppresses it. Therefore long-term, probably life-long treatment is required, all together causing a need for new, safer antiretrovirals (Deeks et al., 2001; Pillay et al., 2000; Powderly, 2002).

The genome of the HIV-particle is encapsulated in a cone-shaped shell comprising about 2000 copies of the capsid protein p24 (Turner and Summers, 1999). The formation of the capsid is essential to the virus, and for this formation to occur correctly, p24 must polymerize, probably to homodimers (Berthet-Colominas et al., 1999; Gamble et al., 1997; Zhang et al., 1996) or possibly hexamers (Li et al., 2000). Single mutations in p24 are sufficient to eliminate polymerization of the capsid (Gamble et al., 1997; Singh et al., 2001), and to inhibit viral replication in culture (Fitzon et al., 2000; Reicin et al., 1995; Tang et al., 2001). Thus, interference with the polymerization of p24 would be an effective new target for anti-retroviral drugs.

Short, chemically modified peptides, corresponding to amino acid motifs in gp120 and p24, such as glycyl-prolyl-glycine-amide (GPG-NH_2), can inhibit the replication of HIV-1 in vitro (Su et al., 2001a). Electron microscopy studies have indicated a possible interaction of GPG-NH_2 with the capsid formation and virus assembly (Hoglund et al., 2002), thus indicating a potential new class of antiretroviral drugs.

Cross-resistance between different drugs of the same class is a common phenomenon (Balzarini et al., 1998; Perno et al., 2001; Pillay et al., 2000). It cannot be excluded that cross-resistance between different classes may occur as well, and this study partially aimed at revealing any cross-resistance between the tripeptide GPG-NH_2 and commercially available drugs. Hence, we studied whether

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mutations related to resistance affect the antiviral activity of GPG-NH_2 by testing 55 clinical HIV-1 isolates exhibiting resistance to licensed drugs for susceptibility to GPG-NH_2 in vitro.

Secondly, the mutations occurring in vitro are often the same as those that occur in vivo (Pillay et al., 2000). Thus, mutations selected for in vitro might predict some, but perhaps not all, mutations that will occur in vivo. The ease with which resistance can be induced, and the mutations correlating to that resistance, might consequently have implications for the possible in vivo use of GPG-NH_2. We therefore investigated whether resistance to GPG-NH_2 can be induced in vitro.

2. Materials and methods

2.1. Cells and virus

Peripheral blood mononuclear cells (PBMCs) from healthy blood donors were purified by Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) density gradient centrifugation and stimulated with phytohemagglutinin (PHA, 2.5 μg/ml, Becton Dickinson Microbiology Systems, Sparks, MD) for 3–5 days. The cells were then cultured in RPMI 1640 medium (Gibco, Paisly, UK) supplemented with 10% heat-inactivated fetal calf serum (FCS, Sigma, St. Louis, MO), 0.1% penicillin/streptomycin (AstraZeneca, Södertälje, Sweden/Sigma, Steinheim, Germany), interleukin-2 (200 iu/ml, Proleukin, Chiron, Amsterdam, The Netherlands), hydrocortisone (5 μg/ml, Sigma, St. Louis, MO) and polybrene (2.24 μg/ml, Sigma, St. Louis, MO).

H9 and MT2 cells were cultured in RPMI 1640 medium supplemented with polybrene, antibiotics and 20 or 10% heat-inactivated FCS, respectively. The cells were incubated with 5% CO_2 at 37 °C and 95% humidity. HIV isolation was performed on blood in EDTA. PBMC were purified with Ficoll-Hypaque and cocultered with PBMC from healthy donors. Stocks of HIV-1 isolates were prepared after one passage by collecting supernatants from infected cultures.

The laboratory strain HIV-1^{IIIB} was kindly provided by R.C. Gallo (then at the National Institutes of Health, Bethesda, MD).

2.2. IC_{50} determinations of clinical isolates

The 50% cell culture infective dose ($CCID_{50}$) end point of each clinical isolate was determined by infecting PBMCs (600,000 cells/ml) with serial, 10-fold, dilutions of the virus (in duplicate). The medium was changed on day 7 and the experiment was terminated on day 14; p24 antigen were quantitated, using an *in house* p24 antigen ELISA, essentially as previously described (Horal et al., 1991). Recombinant p24 (Protein Sciences Corp, Meriden, CT) at a fixed concentration served as standard, the detecting limit of the assay being 0.5 ng/ml.

Drug susceptibility of clinical isolates with different patterns of mutations was evaluated essentially according to Japour et al. (1993). PBMCs (600,000 cells/ml) were infected with clinical isolates at 100 $CCID_{50}$ for 2 h at 37 °C, resuspended in fresh medium containing various concentrations (1, 4, 16, 64 and 256 μM) of GPG-NH_2 (Bachem, Bubendorf, Switzerland) and incubated at 37 °C. Infected cultures without GPG-NH_2 served as controls, and each isolate was plated in duplicate. Supernatants were monitored for contents of p24 antigen (using the previously described *in house* ELISA), and when the virus yield reached approximately 80% of the maximal yield (days 5–9) the drug concentration that inhibited the virus replication by 50% (IC_{50}) was calculated by plotting the amount of p24 antigen against drug concentration.

2.3. Selection of resistance

H9-cells (500,000 cells/ml) were infected with the laboratory strain HIV-1^{IIIB} at 100 $CCID_{50}$ and cultured in the presence of GPG-NH_2 or lamivudine (Epivir, GlaxoWellcome, Basingstoke, UK) at an initial concentration of approximately five times the IC_{50} of respective drug. Viral replication was monitored by observation of the cytopathic effect (CPE), i.e. syncytium formation, and cultures showing extensive CPE after 7–10 days were used for further passage. At each passage an aliquot (150 μl) of the cell free supernatant was used to infect fresh H9-cells. At each passage virus was cultured in the presence of the same or increasing concentration of drug, and supernatants from the cultures with the highest drug concentration showing extensive CPE were used. Since the toxic concentration of the drugs were more than 1000 times the IC_{50}-value, a selection pressure was maintained even in the presence of increased drug concentrations (Soudeyns et al., 1991; Su et al., 2001a). As a control, virus cultured in drug-free medium was passaged in parallel. The titers of each passage, as well as the IC_{50} for each drug, were determined retrospectively using H9-cells. To avoid any inter-test variations, the laboratory strain and the complementary untreated control were analyzed at every IC_{50}-test. The IC_{50} for each passage was thus always compared with the IC_{50} for the laboratory strain. Resistance was defined as more than 10-fold increase in IC_{50} values and confirmed by gene sequencing of the RT, protease, or p24 gene.

To ensure that the GPG-NH_2-treated virus maintained CXCR4 coreceptor usage, the ability to form syncytia in MT2-cells was tested. Aliquots (100 μl) of cell-free supernatant from passage 27 and 30 as well as the laboratory strain used for selection were added to the cells (500,000 cells/ml). The cells were examined for syncytium formation during 14 days.

The growth capacity of the passaged virus was compared with the laboratory strain in H9-cells infected with 100 $CCID_{50}$ of virus. Concentrations of p24 were measured every second day and plotted in a growth curve.

2.4. Sequencing

Nested RT-PCR was used to amplify the RT and protease genes. The outer primer pair 5′CTGTACCAGTAAAATTAAAGCCA and 5′TATGTCATTGACAGTCCAGCT and the inner primer pair 5′ATGGCCATTGACAGAAGAAA and 5′AGGCTGTACTGTCCATTTAT were used for the RT gene. The outer primer pair 5′CCGATAGACAAGGAACT and 5′TCTACTAATGCTTTTATTTTT and the inner primer pair 5′ACTTCCCTCAGATCACTCTT and 5′TTCCTGTCTTTAATTTTACT were used for the protease gene. The p24 gene was amplified with a non-nested system using two overlapping sets of primers; 5′CACAGCAATCAGGTCAGCCAA, 5′CTCCTACTGGGATAGGTGGATT and 5′TACTAGTACCCTTCAGGAACAAAT, 5′CTTGGCTCATTGCTTCAGCCAAA. Sequencing was performed with an ABI PRISM 310 Genetic Analyzer, Applied Biosystem, using the ABI Prism Rhodamine Dye Terminator Cycle Sequencing Ready Reaction kit (PE Biosystems, Warrington, UK). In a bilateral reaction, the inner primers for RT and protease and the two sets of primers for p24 were used for sequencing. Drug resistance-related mutations were defined according to European guidelines (Vandamme et al., 2001).

3. Results

3.1. IC_{50} determinations of clinical isolates

To study whether any cross-resistance may exist between the tripeptide GPG-NH_2 and commercially available drugs, we examined the correlation between mutations related to NNRTI-, NRTI- and PI-resistance and the antiviral activity of GPG-NH_2. Clinical HIV-1 isolates (55 isolates) exhibiting resistance (with various patterns of mutations) to licensed drugs were tested for susceptibility to GPG-NH_2 in PBMCs.

IC_{50} values for GPG-NH_2 ranged from 2 to 37 μM. No correlation between mutations in the RT or protease genes and responsiveness to GPG-NH_2 was found for the clinical HIV-1 isolates tested (Table 1). Neither did the number of mutations influence the responsiveness to GPG-NH_2 (data not shown). One wild-type isolate was tested seven times to reveal the inter-test variation, and the IC_{50}-values ranged from 17 to 34 μM with a mean value of 26.6 ± 6.0 μM.

Table 1
IC_{50} values (GPG-NH_2) for clinical HIV-1 isolates with different mutations in the reverse transcriptase (RT) and protease (P) genes

Mutation	Number of isolates[d]	Mean[e] IC_{50} (μM)	Range IC_{50} (μM)
NRTIs[a]			
RT: T215Y/F	27	16	2–36
RT: K70R	13	17	3.7–36
RT: M184V	28	20	2–37
RT: L74V	4	12	2–33
RT: T69D	5	17	10–29
NNRTIs[b]			
RT: K103N	3	13	2–33
RT: Y181C	3	21	16–26
Pis[c]			
P: G48V	1	16	16
P: L90M	6	18	5.5–33
P: V82A/T	8	16	8–33
P: M46I/L	5	17	9.9–32
≥2 secondary, no primary mutations	23	17	2–36
No primary mutations	15	14	5.3–35
Total	55	17	2–37

(a) Nucleoside analogue reverse transcriptase inhibitors; (b) non-nucleoside reverse transcriptase inhibitors; (c) protease inhibitors; (d) one isolate may have several mutations, and may thus occur in the table several times; (e) geometric mean.

3.2. Selection of resistance

Types of mutations as well as the time span for development of resistance generally correlate in vitro and in vivo; therefore we investigated whether resistance to GPG-NH_2 could be induced in vitro. H9-cells were infected with HIV-1^{IIIB} at 100 $CCID_{50}$ and cultured in the presence of GPG-NH_2 or lamivudine. To determine the inter-test variation in H9-cells, IC_{50} evaluations of HIV-1^{IIIB} were performed on several occasions. IC_{50} values ranged from 7.4 to 36 μM ($n = 9$) with a mean value of 18.6 ± 9.4 μM. Resistance was defined as more than 10-fold increased IC_{50} values and confirmed by gene sequencing of the RT, protease, or p24 gene. Two independent series of selection with GPG-NH_2 were performed, and no increase in IC_{50} was found after 30 passages (Table 2). However, the mutation T107I was detected in the p24 gene at passage 22. No such mutation was found in the untreated controls. After six passages with lamivudine the IC_{50} increased almost 100,000-fold and a well-known mutation in the RT gene occurred (M184V).

To ensure that the GPG-NH_2-treated virus maintained CXCR4 coreceptor usage, the ability to form syncytia in MT2-cells after 30 passages was confirmed. Also, the growth capacity of the GPG-NH_2-treated virus and the untreated control was analysed, showing no increase in growth capacity after 30 passages (data not shown).

4. Discussion

In this report, we found that mutations in the RT or protease gene conferring NRTI-, NNRTI- or PI-resistance did not alter the efficacy of GPG-NH_2 in vitro. Furthermore, even after 30 in vitro passages in the presence of the drug no resistance to GPG-NH_2 was evident. In contrast, resistance to lamivudine was selected after 6 passages.

Recombinant techniques for phenotypic resistance testing are recommended in clinical practice. Such assays are commercially available and are more common than assays based

Table 2
Occurring mutations and IC_{50} values for GPG-NH_2 and lamivudine under selective pressure of respective drug

GPG-NH_2 series 1							GPG-NH_2 series 2							Lamivudine						
Passage number	Day	Concentration (μM)	Mut[a] p24	IC_{50} (μM)	IC_{50} control[b] (μM)	$TCID_{50}$	Passage number	Day	Conc entration (μM)	Mut[a] p24	IC_{50} (μM)	IC_{50} control[b] (μM)	$TCID_{50}$	Passage number	Day	Concentration (μM)	Mut[a] RT	IC_{50} (μM)	IC_{50} control[b] (μM)	$TCID_{50}$
0	0	0	wt[c]	19[d]	19[d]	10^2	0	0	0	wt[c]	19[d]	19[d]	10^2	0	0	0	wt[c]	19×10^{-3}	19×10^{-3}	10^2
1	13	100				10^3	1	10	50				10	1	10	0.1				10^2
2	25	100		9.5		10^4	6	48	100				10^4	2	17	0.2				10^4
4	48	200				10^3	7	55	200				10^3	3	25	0.4		43×10^{-3}		10^3
5	62	250		8.5		10^4	8	64	300		8		10^4	4	33	1.6				10^3
7	95	500		8.3		10^4	19	166	300	wt[c]	36	29	10^3	5	40	10				10^4
8	105	250				10^4	20	174	500				10^3	6	48	40	M184V	$>10^6$		10^3
19	201	400				10^2	22	193	500	T107T/I	64	9.2	10^4	7	55	500				10^4
22	229	400	T107T/I	14	9.4	10^2	23	202	700				10^3	8	64	1000	M184V	$>10^6$	26×10^{-3}	10^3
29	293	600				10^3	25	224	900				10^5	9	74	1000				10^3
30	303	1000	T107I	33	31	10^4	27	240	1300	T107T/I	33	3.7	10^4							
							30	269	1300	T107T/I	18	23	10^4							

Only passages where the drug concentration was altered or susceptibility was tested are shown. Values not shown were not determined. (a) For each selective series the protease, reverse transcriptase and p24 genes were sequenced, but only the genes with occurring mutations are indicated. (b) Control cultures had no drug added. (c) Wild-type. (d) The IC_{50} value for GPG-NH_2 at passage 0 is a mean value from nine tests.

on replication in PBMCs because they are less cumbersome and more reproducible (Hirsch et al., 2000; The Euro-Guidelines Group for HIV Resistance, 2001; Vandamme et al., 2001). However, the recombinant assays are only available for protease- and RT-inhibitors where the sites of mutations generating resistance are known. Furthermore, assays based on replication in PBMCs more closely represent the in vivo situation. We therefore used the PBMC-technique to evaluate whether known resistance-related mutations in clinical isolates affected the efficacy of GPG-NH_2. The cut-off values between susceptible and less susceptible virus are set to a 2.5- to 4-fold increase in IC_{50} in the different commercial recombinant assays, although the clinical significance of an increased IC_{50} of such a magnitude has only been reported for a few drugs (Richman, 2000). Furthermore, 2.5- to 10-fold difference in susceptibility to some drugs may represent wild-type variation as has been reported by Little et al. (1999). The data above and the fact that a larger inter-test variation can be seen with IC_{50} evaluations in PBMC have prompted us to set the cut-off for resistance to a 10-fold increase in the IC_{50} values.

Cross-resistance can occur between different drugs, usually of the same class. No such correlation between resistance-related mutations and susceptibility to GPG-NH_2 was found in this study, supporting earlier findings that GPG-NH_2 has a different mode of action than current available drugs (Hoglund et al., 2002; Su et al., 2001b). Since the variability of the tested isolates is greater than the variability of the control isolate (2–37 μM versus 17–34 μM), it cannot be excluded that there may be differences in susceptibility to GPG-NH_2 between different strains. However, any such heterogeneity does not seem to depend on resistance to other classes of drugs.

After selective drug pressure in vitro, HIV-1 resistant to all classes of commercially available drugs has been demonstrated (Dianzani et al., 1993; Larder et al., 1991; Markowitz et al., 1995; Richman et al., 1991; Tisdale et al., 1993). Depending on the complexity of mutations needed, the time for selection vary. For example, resistance to lamivudine (M184V) can be induced after a few passages (Tisdale et al., 1993), whereas resistance to ritonavir (M46I, L63P, A71V, V82F, I84V) can take more than 20 passages (Markowitz et al., 1995). Here, we tried to select GPG-NH_2-resistant mutants in vitro, which could give an indication on how easily resistance would occur in vivo. Furthermore, localisation of the site of any mutations referring to resistance would strengthen the proposed mode of action of GPG-NH_2. Complete resistance to lamivudine (M184V, 100.000-fold reduced susceptibility) was evident after six passages. After 30 passages in two parallel series, no reduced susceptibility to GPG-NH_2 was found. However, in both series one mutation in the p24 gene (T107I) was found for the GPG-NH_2-treated samples but not for the controls. The fact that this mutation persisted during several passages emphasizes that the mutation might be of importance. Threonine at position 107 is part of a T-cell epitope as well as the binding-site for cyclophilin A and not known to be a site for polymorphism (Colgan et al., 1996; Iroegbu et al., 2000; Seibert et al., 1995; Protein sequence database, 2002). After 30 passages the concentrations of GPG-NH_2 were increased to high amounts and still progeny virus was yielded, although the passaged virus did not show a reduction in its sensitivity towards GPG-NH_2 as measured in the in vitro antiviral assays. This may be due to increased viral fitness, but neither coreceptor usage nor growth capacity of the passaged virus changed.

Perhaps the T107I-mutation is a secondary mutation, which may favour resistance development without directly affecting drug efficacy. Even longer series of selection must be performed to address this possibility, and the possibility of resistance development at a later time point cannot be excluded. To deduce the importance of the T107I-mutation, cloning of the selected virus and site-directed mutagenesis should be performed. On the other hand, it cannot be excluded that the lack of viral resistance to GPG-NH_2 could imply a direct or indirect involvement of a cellular factor in the antiretroviral mechanism. Even if the T107I-mutation does induce or facilitate resistance, the difficulty by which this mutation is induced indicates a favourable in vivo situation where treatment with GPG-NH_2 may be beneficiary without rapid resistance development.

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