



The Non-peptidic Carbohydrate-binding Pradimicin Antibiotics Inhibit HIV infection, Viral Capture by DC-SIGN, and Viral Transmission and thus May Qualify as Potential Microbicide Candidate Drugs

Jan Balzarini¹, Katrien François¹, Dana Huskens¹, Joeri Auwerx¹, Kristel Van Laethem², Yasuhiro Igarashi³, Toshikazu Oki⁴ and Dominique Schols¹

¹Laboratory of Virology and Chemotherapy, ²Laboratory of Clinical and Epidemiological Virology, Rega Institute for Medical Research, K.U.Leuven, Leuven, Belgium; ³Biotechnology Research Center, Toyama Prefectural University, Toyama, Japan; ⁴Keck School of Medicine, University of Southern California, Los Angeles, USA

Rega Institute for Medical Research
Katholieke Universiteit Leuven
B-3000 Leuven, Belgium
Dominique.Schols@rega.kuleuven.be
Phone: +32-16-337373
Fax: +32-16-337340

BACKGROUND

It has been shown that DC-SIGN, expressed by dendritic cells, promotes efficient HIV infection in *trans* of CD4⁺ cells. The design and development of candidate microbicide drugs have to be taken into account for this mechanism of dissemination of the incoming virus. The outcome of this type of studies would be very helpful to guide the choice of potential candidate microbicide drugs. Several of the carbohydrate-binding agents (CBAs) are shown to be potential drug candidates for microbicial use (see also poster #747 and J. Balzarini, Nat.Rev. Microbiol. 5,583-597, 2007).

METHODS

Test compounds. The mannose-specific plant lectins from *Galanthus nivalis* (GNA), *Hippeastrum hybrid* (HHA), *Narcissus pseudonarcissus* (NPA) and the GlcNAc-specific plant lectin from *Urtica dioica* (UDA) were kindly provided by Dr. E. Van Damme (Ghent University, Belgium). Pradimicin A (PRM-A) (MW:838) was isolated from the fermentation broth of actinomycete sp. TP-A0016. PRM-S (MW:951) is a sulfated pradimicin analog isolated from *A. spinosa* AA0851 (from Dr. Y. Igarashi).

Antiretrovirus assays. Primary clinical isolates representing different HIV-1 clades (Table 1) were all kindly provided by Dr. J. Laethem from SeraCare Life Sciences, Milford, and their co-receptor use (RS or X4) was determined. PBMC from healthy donors were stimulated with PHA for 3 days and then seeded at 0.5 x 10⁶ cells per well into a 48-well plate containing varying concentrations of compound and IL-2. The virus stocks were added at a final dose of 250 pg p24/ml. Cell supernatant was collected at day 10-12 and the culture supernatant was analyzed by p24 Ag ELISA. C8166 cells (5 x 10⁵ cells per ml) were suspended in culture medium and infected with HIV-1 at 100 CCID50 per ml. Then, 100 µl of the infected cell suspension were transferred to the wells, mixed with 100 µl of the test compounds, and further incubated at 37°C. After 3 to 4 days, C8166 giant cell formation was recorded microscopically (condition A, Table 3).

Effect of continuous presence of test compounds on syncytia formation in co-cultures of virus-exposed Raji/DC-SIGN cells and uninfected C8166 cells. Raji/DC-SIGN cell cultures were exposed to HIV-1 for 30 min to allow virus capture and subsequently thoroughly washed with culture medium as described above. Then, virus-exposed Raji/DC-SIGN cells were seeded in 96-well microtiter plates in the presence of serial dilutions of the test compounds. Immediately after seeding, C8166 cells were added to each well. After 36 to 48 hrs, syncytia formation was recorded microscopically (condition B, Table 3).

Effect of short exposure of HIV-1 to test compounds on prevention of HIV-1-capture by Raji/DC-SIGN cells. High amounts of HIV-1 particles were exposed to serial dilutions of the test compounds for 30 min. Then, the drug-exposed virus suspensions were mixed with Raji/DC-SIGN cell suspensions for 60 min at 37°C after which the cells were thoroughly washed. The Raji/DC-SIGN cell cultures were then analysed for p24 content (data in Table 2, Fig. 1), or mixed with 2 x 10⁵ C8166 cells and further incubated in 48-well plates for 36-48 hrs at 37°C. Then, the syncytia formation in the cell cultures was recorded microscopically (condition C, Table 3).

RESULTS

Inhibitory activity of PRM-A and PRM-S on HIV-1 strains and clinical HIV-1 clades in PBMCs. PRM-A and PRM-S were endowed with an equally and consistent suppressive activity against a wide variety of HIV-1 isolates (independent on their co-receptor use) in PBMCs (Table 1). The mannose-specific plant lectins (such as HHA and GNA) showed a much higher variability in their virus-suppressive potential [i.e. 1.2-1.9 µg/ml (clade O) to 40- >100 µg/ml (clade C)]. In addition, both compounds are also active against various SIV and HIV-2 strains. The CC₅₀ for PRM-A and PRM-S in PBMCs are >250 µg/ml.

Inhibitory effect of test compounds on the ability of Raji/DC-SIGN to capture HIV-1 particles. The CBA dose-dependently inhibited capture of HIV-1 by the Raji/DC-SIGN cells. In contrast, none of the other classes of HIV entry inhibitors were markedly inhibitory at the concentrations tested. The polyanions did not inhibit HIV capture by the Raji cells. Instead, some of these compounds (such as PRO-2000) stimulated HIV capture by ~ 2- to 3.5-fold (Fig. 1, Table 2).

Co-cultivation of T-lymphocyte C8166 cells and HIV-exposed Raji/DC-SIGN cells. In co-cultures of uninfected C8166 cells and HIV-1-exposed Raji/DC-SIGN cells, an abundant amount of syncytia were formed at 36 hrs post-cultivation. This assay system is useful to evaluate the inhibitory activity of the entry inhibitors on HIV transmission.

Inhibitory effect of entry inhibitors on syncytia formation in co-cultures of C8166 cells and virus-exposed Raji/DC-SIGN cells. In a first set of experiments, the entry inhibitors were administered to HIV-1-infected C8166 cells at the time of infection. Different antiviral potencies were observed depending the nature of the entry inhibitor (Table 3, first column). In a second set of experiments, the test compounds were added to the co-cultures of uninfected C8166 cells and Raji/DC-SIGN cells that were pre-exposed to HIV and were kept present throughout the cell culture incubation period (Table 3, second column). Most compounds had higher EC₅₀ values for giant cell formation in these C8166 + HIV-1-exposed Raji/DC-SIGN co-cultures than in HIV-1-infected C8166 cell cultures. In a third set of experiments, the entry inhibitors were only exposed to HIV-1 for a short time period (30 min) after which the drug-exposed virus was administered to the Raji/DC-SIGN cells for 60 min. Then, C8166 cells were added (Table 3, third column). Most CBAs showed a pronounced dose-dependent inhibitory potential.

Table 1. Inhibitory activity of CBAs on several HIV strains and clinical HIV-1 isolates (# clades) in PBMCs

CBAs	EC ₅₀ (µg/ml)										
	A	B	C	D	E	G	O	NI.43	IIIa	IIIb	IIIc
UG273 (RS)	(US)	ETH2220 (RS)	UG270 (X4)	ID12 (RS)	BZ163 (RS)	BCF ^a (RS)	BCP06 (X4)	(X4)	(X4)	(RS)	
PRM-A	4.1	2.3	8.7	3.1	3.4	11	2.2	1.5	0.69	2.8	2.2
PRM-S	14.2	15.1	15.6	8.9	4.5	8.9	15.4	4.4	4.5	7.7	5.3
GNA	27	17	≥ 100	> 20	19	25	≥ 50	1.9	1.4	0.33	6.5
HHA	29	5.4	44	4.9	12	4.6	41	1.2	0.35	1.1	6.0

^a50% Effective concentration required to inhibit HIV replication in PBMC cell cultures.

DISCUSSION

Only CBAs are able to efficiently prevent virus transmission from virus-captured DC-SIGN⁺ cells to uninfected T cells. None of the other entry inhibitors proved very effective in interrupting the HIV transmission process. It was even observed that the polyanions, especially PRO-2000, stimulated HIV-1 capture by DC-SIGN⁺ cells (Fig. 1). However, several of its members, PRO-2000 (and also cellulose sulfate (CS), Usher gel) are currently subject of clinical microbicide studies. The stimulatory properties of the polyanions on HIV capture and subsequent transmission found in our assay system – if physiologically relevant – is not recommended to occur in the presence of a potential microbicide candidate drug. A potential disadvantage of using natural products as HIV therapeutics is their protein nature. It is indeed not technically easy and relatively costly to produce and purify such protein CBAs at large scale; they may have poor, if any, oral bioavailability and they may also trigger an immune response when administered systemically at a frequent application basis. Although general *in vivo* toxicity should be a realistic concern with the CBA therapeutic concept it should be mentioned that in our previous studies *in vivo* injection of CBAs (i.e. HHA, GNA, UDA) in mice did not result in acute animal toxicity. Also, a PRM-A derivative was shown to have *in vivo* antifungal activity in the treatment of experimental pulmonary aspergillosis in persistently neutropenic rabbits. In this study, the drug was well tolerated at all dosing schedules (up to 150 mg/kg/day) and no toxicity was noted (Ueki, T., Oka, M., Fukagawa, Y. & Oki, T.J. *Antibiotics* 46, 465-477, 1993). Moreover, it was shown that the PRM-A derivative can only bind to terminal D-mannopyranosides of glycans that are abundantly present on HIV gp120, whereas normal animal cells show no binding, probably because D-mannopyranoside terminals which are necessary for PRM-A binding are very scarce on the cell surface. Interestingly, large doses of PRM-A have been administered to animals in the past, with minimal toxicity (Walsh, T.J. & Giri, N. *Eur. J. Clin. Microbiol. Infect. Dis.* 16, 93-97, 1997), and the compound has already been subject to phase I/II clinical trials for its anti-fungal activity. Due to its lipophilic properties, PRM-A has a limited water-solubility. However, a novel sulfated derivative, designated PRM-S, has a much higher solubility, which may therefore have advantages from a therapeutic viewpoint.

Table 2. Effect of test compounds on the ability of Raji/DC-SIGN cells to capture HIV-1(IIIa) particles

Test compound	IC ₅₀ ^a (µg/ml)
HHA	8.5
GNA	18
NPA	0.7
PRM-A	2.1
PRM-S	11
UDA	10
mAb 2G12	0.80
T-20	> 25
AMD3100	> 50
DS-5000	> 250
PVAS	> 250
PRO-2000	> 25

^a50% inhibitory concentration or compound concentration required to prevent HIV-1 capture (adsorption) by Raji/DC-SIGN cells as measured by HIV-1 p24 ELISA.

Table 3. Inhibitory activity of test compounds against HIV-1-infected C8166 cells or HIV-1-exposed Raji/DC-SIGN cells cocultivated with uninfected C8166 cells

Test compound	Condition [A]	Condition [B]	Condition [C]
	EC ₅₀ ^a (µg/ml) (HIV-1-infected C8166 cells)	EC ₅₀ ^b (µg/ml) (continuous presence of test compound in the Raji/DC-SIGN + C8166 co-culture after virus exposure to Raji/DC-SIGN cells)	EC ₅₀ ^c (µg/ml) (virus pre-incubated with test compound prior to exposure to Raji/DC-SIGN)
HHA	13 ± 6.5	> 20	32 ± 7.5
GNA	20 ± 0.0	> 20	42 ± 2.5
NPA	0.90 ± 0.1	3.1 ± 0.9	0.9 ± 0.3
PRM-A	2.5 ± 1.7	2.0 ± 0.2	-
PRM-S	6.7 ± 2.3	4.4 ± 1.4	-
UDA	25 ± 15	45 ± 5.0	30 ± 10
mAb 2G12	2.0 ± 0.0	22 ± 2.5	0.9 ± 0.1
T-20	0.75 ± 0.05	2.7 ± 1.2	> 25
AMD3100	0.035 ± 0.015	0.85 ± 0.65	> 50
DS-5000	0.8 ± 0.0	4.8 ± 3.2	> 250
PVAS	1.4 ± 0.6	2.2 ± 0.25	≥ 250
PRO-2000	0.3 ± 0.1	6.0 ± 4.0	≥ 25

^a50% effective concentration, required to prevent syncytia formation in HIV-1(IIIa)-infected C8166 cell cultures at day 3 post infection.

^b50% effective concentration, required to prevent syncytia formation in HIV-1(IIIa)-exposed Raji/DC-SIGN cell cultures that were cocultivated with uninfected C8166 cells in the continuing presence of different concentrations of the test compounds.

^c50% effective concentration, required to prevent syncytia formation in test compound-pre-exposed HIV-1(IIIa)-infected Raji/DC-SIGN cell cultures, co-cultivated with uninfected C8166 cells in the absence of the test compounds.

CONCLUSIONS

CBAs, but not other classes of HIV entry inhibitors, can efficiently interrupt and prevent HIV particle capture by DC-SIGN-expressing cells. In addition, the non-peptidic pradimicin derivatives have consistent anti-HIV activity and are interesting drug candidates for further preclinical research.

These data are partially published in:
Balzarini, J., Van Herreweghe, Y., Vermeire, K., Vanham, G. & Schols, D. *Mol. Pharmacol.*, 71: 3-11 (2007).
Balzarini, J., Van Laethem, K., Daelaemans D., Hatse S., Bugatti A., Rusnati M., Igarashi Y., Oki T. & Schols D. *J. Virol.* 81, 3622-373 (2007).

The research was supported by the EMPRO Project of the European Commission, the Fondation Dorneur and the Centers of Excellence of the K.U.Leuven.

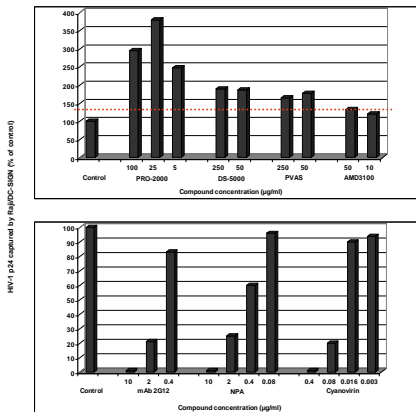


Fig. 1. Effect of various classes of agents on the capture of HIV-1 by Raji/DC-SIGN cells. HIV-1(IIIb) particles were exposed to various dilutions of the agents (30 min) prior to administration to Raji/DC-SIGN cells for 60 min. After removal of unbound virus, by several washing steps, cell-associated virus was quantified by p24Ag ELISA.