

Synthetic theta-Defensins, which Inhibit HIV-1 Entry, Exhibit Different Activity against Primary HIV-2 and SIV Isolates

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a. Abstract

- **Background:** Defensins are endogenous, cysteine-rich peptides that contribute to host defense against microbial and viral infections, and enhance certain adaptive immune responses. Theta (θ)-defensins are tetracyclic peptides with 18 residues and three disulfide bonds. θ -defensin genes and/or peptides have been found in many non-human primates, including Old World monkeys, lesser apes and the orangutan. RTD-1 and RTD-2 are naturally occurring θ -defensins that have been isolated from the leukocytes and bone marrow of Rhesus macaques. Retrocyclin-1 (RC-100) and Retrocyclin-2 (RC-100b) are synthetic θ -defensins whose sequences are found in human θ -defensin pseudogenes. RC-112 and RC-100 have identical sequences, but RC-112 (*enantio*Retrocyclin-1) is composed exclusively of D-amino acids. All of these θ -defensins are lectins that bind various N- and O-linked carbohydrates, and all of them can protect cells from HIV-1 infection.
- **Objective:** to analyze the activity of diverse θ -defensins against primary HIV-2 and SIV isolates.

- **Methods:** Binding studies of RC-100 and RC-100b (retrocyclin-2) to SIVgp130 were done by surface plasmon resonance (SPR). The activity of synthetic RC-100, RC-100b, RC-112, RTD-1 and RTD-2 against selected HIV-2 and SIV isolates was tested in an inhibition assay using the JC53-BL reporter cell line. Statistical comparisons of their IC₅₀ values were done by paired Student t-tests.
- **Results:** θ -defensins did not display consistent activity against most HIV-2 and SIV isolates, even when tested at peptide concentrations much higher than those effective against HIV-1 isolates. By SPR, the affinities (Kd) of RC-100 and RC-100b for SIVgp130 were 151 nM and 117 nM, respectively- much higher than those previously published for HIV-1LAVgp120 (35.4 nM for RC-100, and 9.41 nM for RC-100b). These higher Kd values indicate the formation of less stable θ -defensin/env complexes.
- **Conclusions:** Results from these inhibition assays support previous findings that suggest there are differences between HIV-1 and HIV-2 entry. The reported decrease in binding and inhibition may be due to the different glycosylation patterns observed between HIV-1 and HIV-2/SIV isolates. Furthermore, these results suggest θ -defensins are not likely to be good therapeutics for HIV-2 infections.



b. Background: Defensins

- Are amphipathic, cationic, cysteine-rich endogenous peptides
- Have antimicrobial and antiviral activity and play roles in innate immunity
- Three subfamilies (α , β , and θ) of defensins exist in vertebrates
- They share a common ancestor and have β -sheet structures with three SS bonds
- θ -defensins are the only cyclic peptides known to be produced by any animal
- In humans, mRNA homologous to the rhesus θ -defensin is expressed in bone marrow, but translation is silenced by a premature stop codon mutation
- Synthetic θ -defensins, derived from the human pseudo-gene sequences, prevent the entry of X4 and R5 strains of HIV-1
- Naturally occurring rhesus macaque θ -defensins, RTD-1 and RTD-2, also inhibit the ability of HIV-1 isolates to cause infection

c. Theta-defensins

In nature, each 9-a.a. precursor:

- is the product of 2 mutated alpha-defensin genes with a premature stop codon
- contains 3 cysteines that form the intra-molecular disulfide bonds
- is joined head-to-tail to another precursor by a post-translational mechanism that is not currently understood

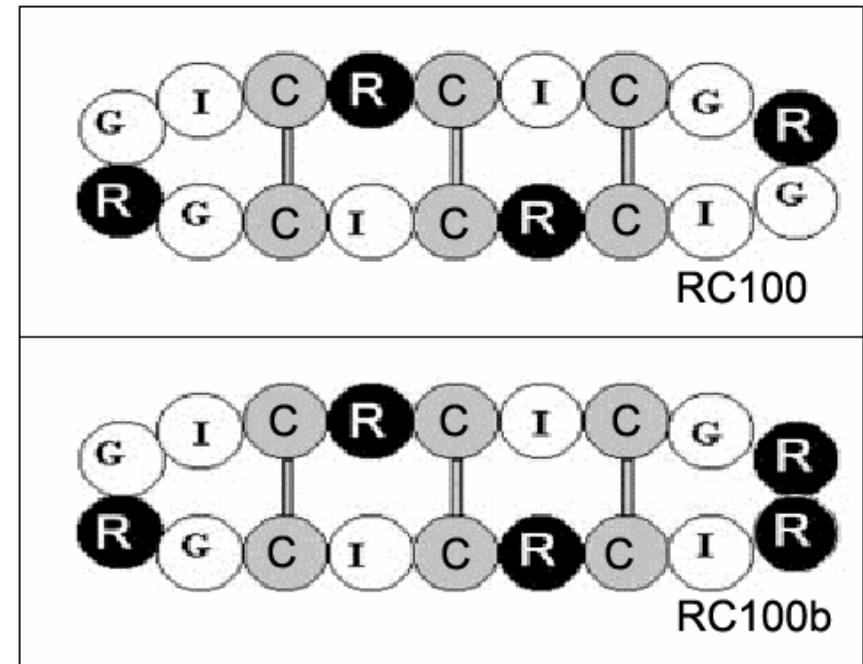
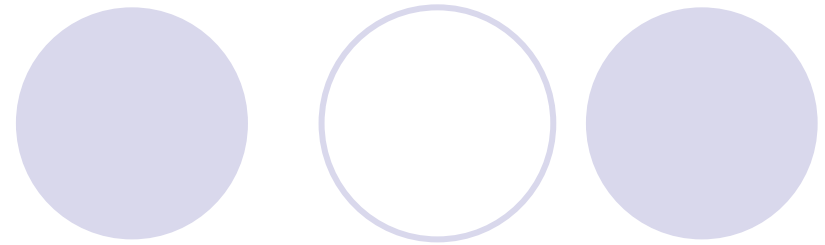
Synthetic peptides (UCLA):

1. synthesized as a linear 18-a.a. precursor
2. oxidized to form the intra-molecular disulfide bonds
3. rendered cyclic

Peptide	Comments	Sequence of linear precursor
RC-100	Retrocyclin-1	GICRCICGRGICRCICGR
RC-100b	Retrocyclin-2	GICRCICGRRICRCICGR
RC-112	<i>Enantio-retrocyclin-1</i> (all D ₁₋₁₈)	GICRCICGRGICRCICGR
RTD-1	Rhesus macaque θ-defensin-1	GFCRCLCRRGVCRCICTR
RTD-2	Rhesus macaque θ-defensin-2	GVCRCCLCRRGVCRCICRR
OTD-2	Orangutan θ-defensin	GVCRCICGRGVCRCICRR

d. Objectives

- to analyze the activity of synthetic θ -defensins against HIV-2 and SIV primary isolates
- to compare peptide binding properties to recombinant HIV-1gp120 and SIVgp130
- to test the activity of the newly synthesized θ -defensin of orangutan origin, OTD-2, against HIV-1, HIV-2, and SIV



e. Characteristics of viral isolates tested

Strain name	type	Origin	Coreceptor usage
BaL	HIV-1	USA	R5
310340b	HIV-1	Ivory Coast	R5
77618	HIV-2	Ivory Coast	R5X4
GB122	HIV-2	Guinea Bisseau	R5X4
SLRHC	HIV-2	Guinea Bisseau	R5
SIV-hu ^a	SIV	sooty mangabey	no R5X4
SIVsm3	SIV	sooty mangabey	R5
SIVst	SIV	stump tailed macaque	R5

a. SIV-hu is the first SIV isolated from a laboratory worker and sequence analysis shows homology to SIVB670, originally isolated from a sooty mangabey. Infections in GHOST cell lines indicated that coreceptors other than R5 and X4 were used for entry into the cells.

f. Materials and methods

- **JC53-BL:**
 - HIV-1 reporter cell line derived from HeLa cells
 - expresses high levels of CD4, CXCR4 and CCR5
 - contains cassettes for luciferase and β -galactosidase, each driven by the HIV-1 LTR
- viral infectivity and titers were measured by luciferase activity and β -galactosidase staining
- **RC-100, RC-100b, RC-122, RTD-1, RTD-2, and OTD-2** were tested against the viral isolates at concentrations of 20, 10, 5, 2.5, 1.25, and 0.625 $\mu\text{g/ml}$
- Peptide cytotoxicity was analyzed by trypan blue-dye exclusion and by a MTT-based cell proliferation kit
- **Surface plasmon resonance (SPR)** experiments for binding to immobilized HIV-1LAVgp120 and SIVsm239gp130 were done in a Biacore 2000 system
- Binding data were analyzed with BIAevaluation 3.1 software

g. Results of binding studies by SPR

Binding to immobilized SIVmac239gp130

Peptide	K_{on} ($\times 10^4$)	K_{off} ($\times 10^{-4}$)	Kd_{SIV} (nM)
Retrocyclin-1 (RC-100)	7.98	121	151
Retrocyclin-2 (RC-100b)	2.92	34.3	117
RC-112	6.55	33.1	50.5
CD-4	1.25	4.74	37.9
Cyanovirin N	0.464	1.95	42

CV-N was included as a control, it is a potent HIV-1 entry inhibitor and it binds selectively to high-mannose oligosaccharides on gp120 with high affinity

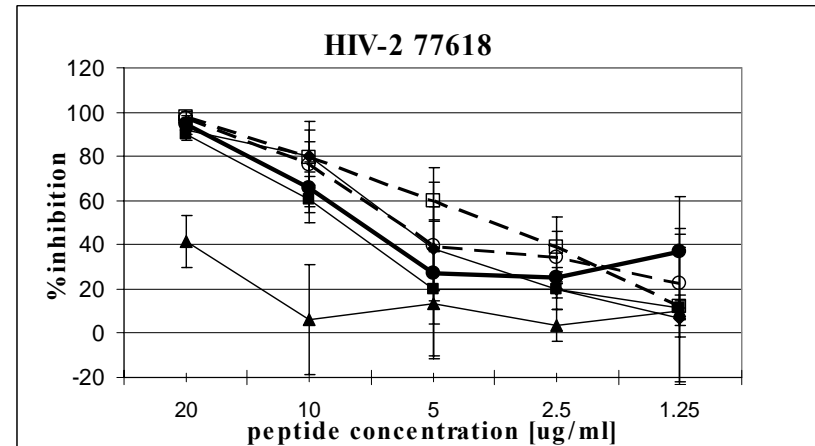
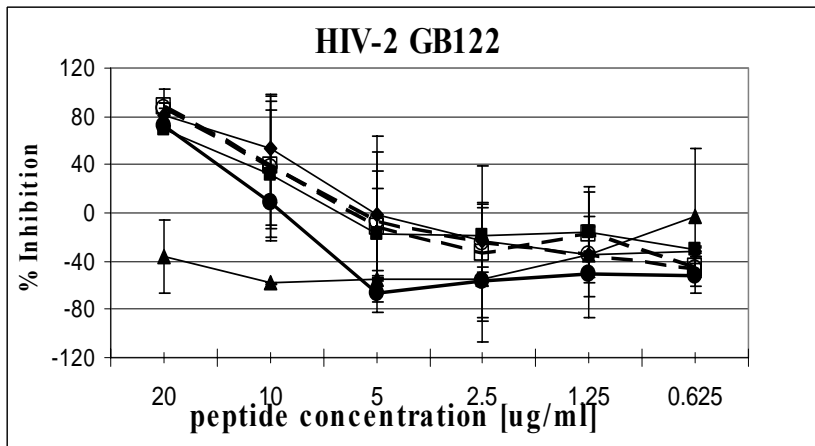
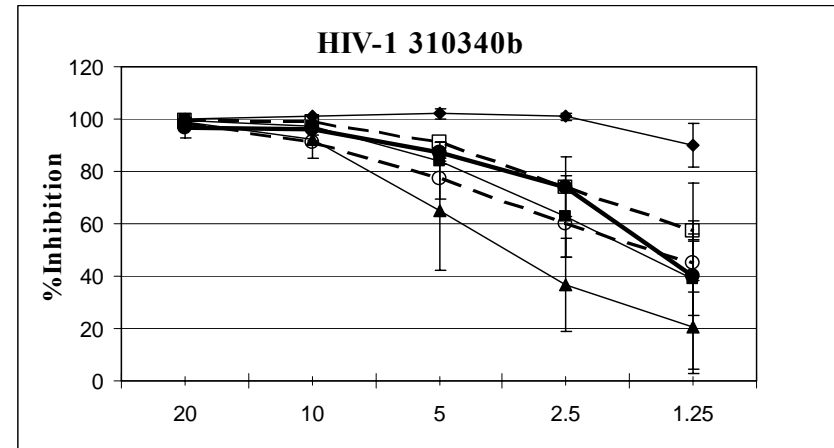
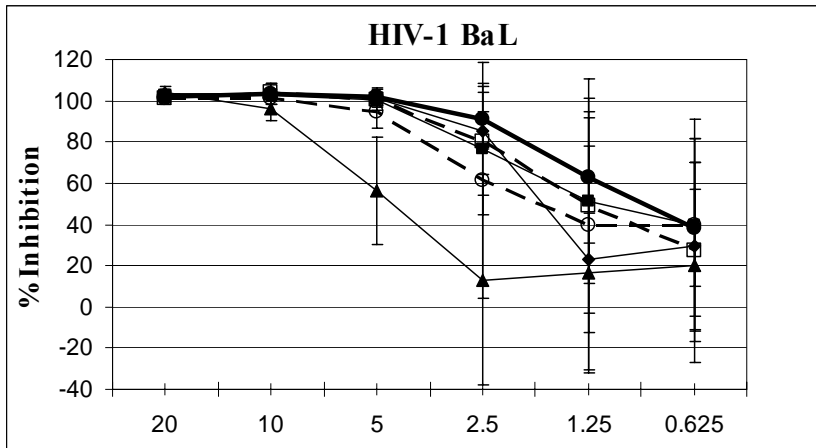
- ✓ **High K_{off} rates indicate less stable complexes between the molecules and SIVgp130**
- ✓ **Binding affinities for gp130: CD4 > CV-N > RC-112 > RC-100b > RC-100**

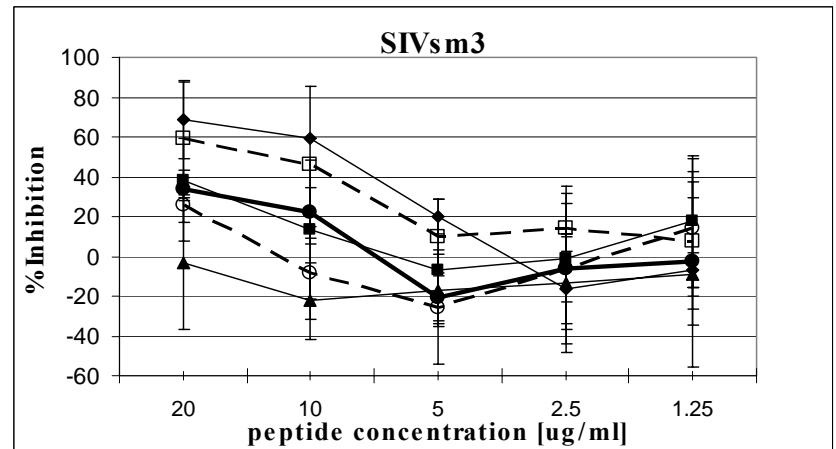
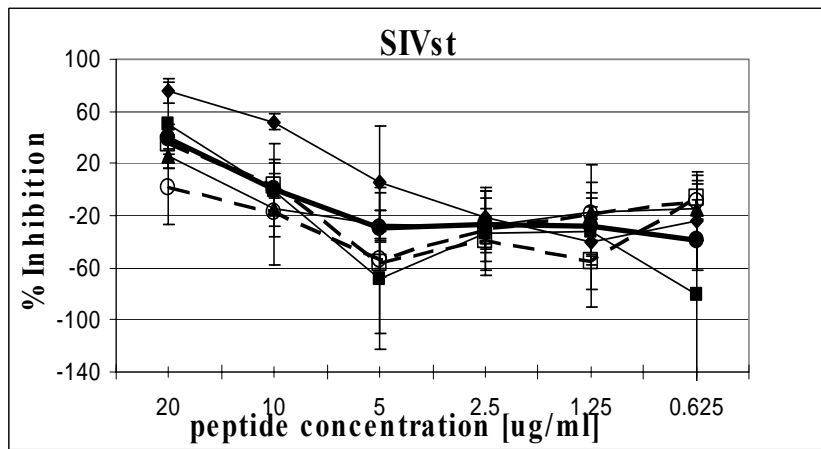
Comparative binding results of θ -defensins to immobilized SIVmac239gp130 and HIV-1LAVgp120

Peptide	$K_{d_{SIV}}$ (nM) Immobil-gp130	$K_{d_{HIV-1}}$ (nM) Immobil- gp120	$K_{d_{SIV}}/K_{d_{HIV-1}}$ Ratio
Retrocyclin-1 (RC-100)	151	35.4	4.27
Retrocyclin-2 (RC-100b)	117	9.41	12.43
CD-4	37.9	10.4	3.64
Cyanovirin N	42	6.60	6.37

- ✓ CD-4 and CV-N form stable complexes with both HIV-1gp120 and SIV gp130 (high affinity)
- ✓ RC-100 and RC-100b bind more avidly to HIV-1LAVgp120 than to SIVgp130
- ✓ RC-100b shows better affinity for both viral glycoproteins

h. Effects of RC-100, RC-100b, RC112, RTD-1, RTD-2 and OTD-2 on HIV-1, HIV-2 and SIV infections





$\% \text{ Inhibition} = 1 - (\text{average RLU peptide} + \text{virus} / \text{average RLU virus alone}) \times 100$

An inactive peptide was included in all the experiments as a negative control

Data presented are the mean \pm standard deviation calculated from duplicate or triplicate (77618, SIVst) experiments in the reporter cell line JC53-BL

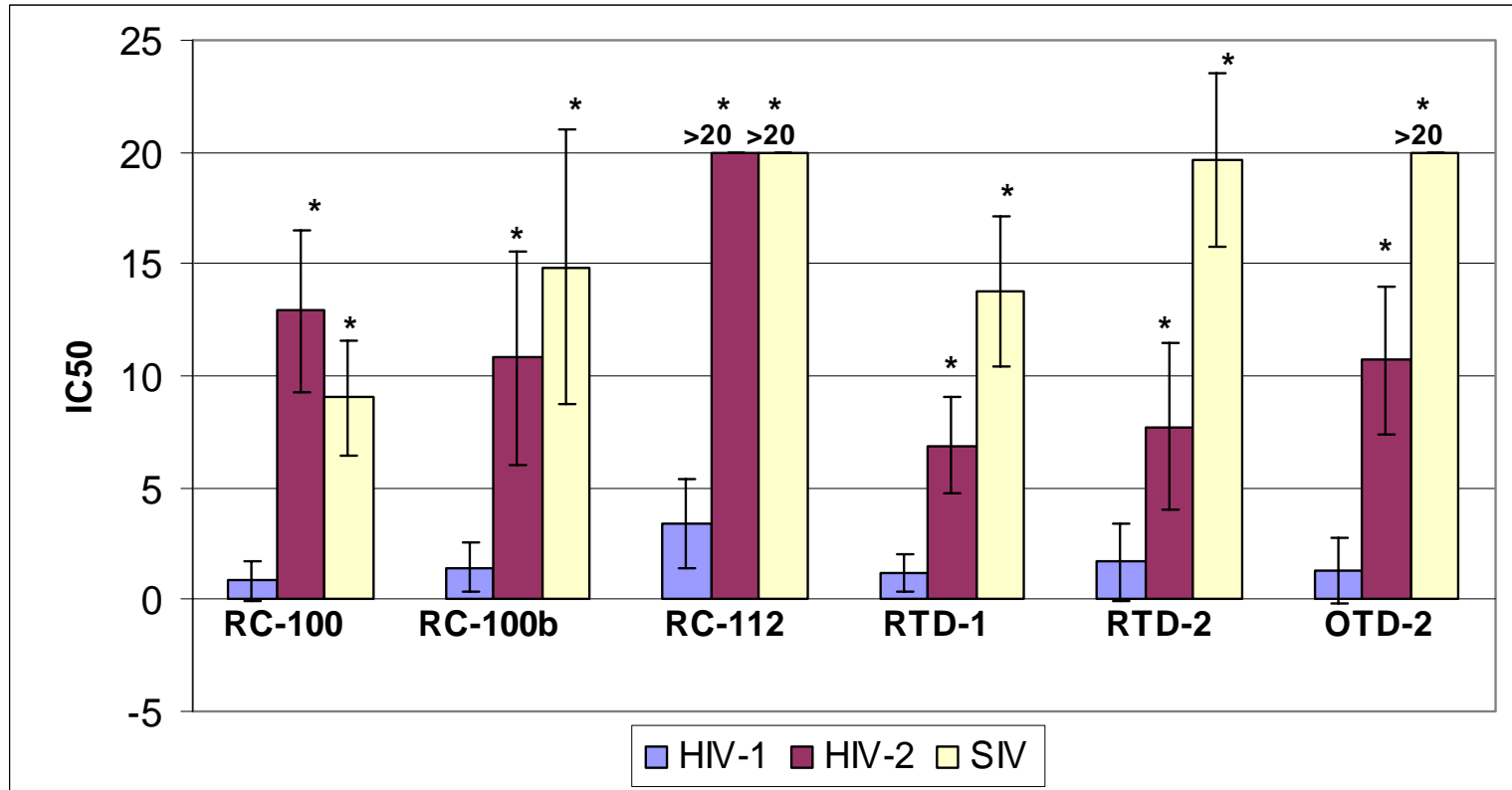
- ✓ **HIV-1 isolates are highly inhibited by all the synthetic peptides at 10 $\mu\text{g/ml}$ (>90 % inhibition)**
- ✓ **HIV-2 and SIV strains are less consistently inhibited by RC-100 and RC-100b even at 20 $\mu\text{g/ml}$ peptide concentration**
- ✓ **RC-112 (*enantio*RC100) is not effective at inhibiting HIV-2 and SIV isolates, while it shows great inhibitory effect on HIV-1 infections**

i. Summary of IC₅₀ ± standard deviation

Strain name	IC ₅₀ (µg/ml) ± standard deviation (SD)					
	RC100	RC100b	RC112	RTD-1	RTD-2	OTD-2
BaL	1.64 ± 1.68	1.20 ± 1.35	3.38 ± 2.40	1.63 ± 1.56	1.79 ± 2.47	0.98 ± 1.22
310340b	0.04 ± 0.06	1.69 ± 0.91	3.43 ± 1.62	0.71 ± 0.15	1.55 ± 0.92	1.52 ± 1.70
77618 ¹	5.41 ± 1.28	6.80 ± 2.87	>20	4.76 ± 3.33	4.81 ± 2.78	5.06 ± 0.35
GB122	13.18 ± 9.64	11.40 ± 4.52	>20	8.06 ± 2.53	11.30 ± 6.70	13.43 ± 0.54
SLRHC	>20	14.16 ± 6.95	>20	7.83 ± 0.65	7.11 ± 1.64	13.65 ± 9.00
SIV-hu ^{a1}	7.56 ± 3.72	11.16 ± 8.00	>20	7.72 ± 0.91	19 ± 11.65	>20
SIVsm3	10.06 ± 1.45	16.88 ± 4.40	>20	13.54 ± 9.13	>20	>20
SIVst ¹	9.49 ± 2.48	16.50 ± 6.07	>20	>20	>20	>20

1. IC₅₀ were calculated from three different experiments, the remaining isolates were done in duplicates

j. Statistical analysis by paired Student t-test



1. Student t-test was calculated with a 95% confidence interval

* p-values show all the differences found between HIV-1 and HIV-2 or SIV

infections in the presence of the synthetic peptides were statistically significant

k. Conclusions



- ❖ **All of the synthetic θ -defensins, including those of rhesus macaque and orangutan origin, inhibited infection of the JC53-BL cells by HIV-1**
- ❖ **The high IC_{50} values of RC-100 and RC-100b confirm their reduced efficacy against HIV-2 and SIV, relative to HIV-1**
- ❖ **Synthetic θ -defensins bound recombinant SIVmac239gp130 with lower affinity than recombinant HIV-1 gp120, however, the significance of this finding is uncertain because of the variability in binding affinities previously observed with recombinant HIV-gp120 from different sources**

- ❖ **HIV-2 isolates were more susceptible than SIV isolates to RTD-1, RTD-2, and OTD-2, however, they were less susceptible than HIV-1**
- ❖ **Despite strong binding to SIVmac239gp130 ($K_d = 50.5$ nM), RC-112 had little protective effect on HIV-2 or SIV infections ($IC_{50} > 20$ µg/ml). Since RC-112 is *enantio*RC-100, some chiral interaction(s) between SIV/HIV2 and the JC53-BL target cells may be more important for these viruses than for HIV-1**
- ❖ **θ- defensins are not likely to be effective therapeutics for HIV-2 infections, despite their considerable potential for HIV-1**