Quinoxapeptins: Novel Chromodepsipeptide Inhibitors of HIV-1 and HIV-2 Reverse Transcriptase

I. The Producing Organism and Biological Activity

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Quinoxapeptin A and B are novel chromodepsipeptides which were isolated from a nocardioform actinomycete with indeterminant morphology. Quinoxapeptins A and B are potent inhibitors of HIV-1 and HIV-2 reverse transcriptase and almost equally active against two single mutants forms as well as a double mutant form of HIV-1 reverse transcriptase. Quinoxapeptin A and B are specific inhibitors of HIV-1 and HIV-2 reverse transcriptase because they did not inhibit human DNA polymerase α, β, γ and δ. Quinoxapeptin A and B are structurally similar to luzopeptin A which was also active against HIV-1and HIV-2 reverse transcriptase.

An essential step in the life cycle of the HIV-1 and HIV-2 viruses is reverse transcription of the viral RNA genome to produce a double-stranded DNA copy that is mediated by a virally encoded reverse transcriptase. Inhibition of reverse transcriptase by 3'-azido-3'-deoxythymidine, dideoxyinosine and dideoxycytosine are clinically effective in treating HIV-1 infection thus establishing reverse transcriptase as a therapeutic target for intervention in the progression of AIDS[1,2]. The advent of resistance by the viruses to the available clinical compounds has provided added impetus to the search and development of drug candidates that are more efficacious against the virus and that do not readily lead to the development of resistance.

In an attempt to find such clinical candidates we initiated screening of microbial extracts for inhibitors of HIV-1 reverse transcriptase. Our efforts resulted in the detection and isolation of two related novel chromodepsipeptides from a nocardioform actinomycete with indeterminant morphology. The compounds, which we have named quinoxapeptin A and B, were structurally similar to luzopeptin A[3~6] and sandriamycin[7,8] the former of which was also active against HIV-1 and HIV-2 reverse transcriptase.

Methods

MA7095 (ATCC 55599) was isolated from a bark disc taken from a specimen of *Betula papyrifera*, at the Denali National Park in Alaska. The bark sample was lyophilized, homogenized and plated on a humic acid-based isolation medium[9]. The culture was isolated from this medium and purified on yeast malt extract agar.

A seed culture was produced by inoculating 50 ml of aqueous medium in a 250 ml triple baffled erlenmeyer flask with vegetative mycelia. The medium contained (in g/liter) glucose, 10; soluble starch, 20; yeast extract, 5; N-Z amine E, 5; $CaCO_3$, 1; beef extract, 3; and Bacto-peptone, 5; in distilled water adjusted to pH 7.0 with NaOH prior to $CaCO_3$ addition and sterilization. Flasks were incubated at 28°C and shaken at 220 rpm for 96 hours in order to obtain sufficient biomass to inoculate the production medium.

The production medium was inoculated by aseptic transfer of 2 ml of seed culture to 44 ml of production medium in a 250 ml non-baffled erlenmeyer flask. The production medium contained (in g/liter) dextrin, 20; beta-cyclodextrin, 10; primary yeast, 10; tomato paste, 20; and $CoCl_2 \cdot 6H_2O$, 0.005; in distilled water adjusted to pH 7.2 with NaOH prior to sterilization. The production flasks were incubated at 28°C and shaken at 220 rpm for 13 days prior to harvest. The contents of individual flasks were pooled and the active compounds

were isolated and characterized as described[10].

Observations of growth, general cultural characteristics and carbon source utilization were made in accordance with published methods[11~16]. Chemical composition of the cells was determined as described[17]. Whole cell fatty acids were derivatized and analyzed as methyl esters (FAMEs) by gas chromatography as described[18] using a MIDI Microbial Identification System (Microbial Identification Systems, Newark Delaware). Tree-based models for classification were created using published methods[19]. Coloration of the culture was determined by comparison with color standards contained in the Inter-Society Color Council-National Bureau of Standards Centroid Color Charts (US Dept. of Commerce National Bureau of Standards supplement to NBS Circular 553, 1985).

Enzyme Assays

HIV-1, HIV-2 reverse transcriptase and DNA polymerase enzyme assays were performed essentially as described[20,21]. HIV-1 mutants were prepared, expressed and purified as described[22]. Extracts and pure samples were dissolved in 100% dimethyl sulfoxide (DMSO) and diluted twenty-fold into the reaction mix such that the final DMSO concentration was 5%.

Results

Chemotaxonomic Characteristics

The peptidoglycan of MA7095 contained *meso*-diaminopimelic acid. The whole cell sugars contained rhamnose, mannose and madurose in trace amounts. Major whole cell fatty acids are listed in Table 1.

General Growth Characteristics

Good to excellent growth was observed on yeast-extract malt-extract agar, sucrose-nitrate yeast extract agar, oatmeal agar and trypticase soy broth agar. Fair to moderate growth was observed on inorganic salts starch agar, tap water agar and CZAPEK's agar. Growth occurred at both 27 and 37°C.

Colony Morphology

On yeast-malt agar at 21 days, the substrate mycelium was a grayish red-brown. Colonies were opaque, raised, with an entire edge and matte surface. The colonies were rubbery in texture and aerial mycelia were not observed.

Micromorphology

Substrate mycelia measured 0.76~1.14 μm diameter and showed moderate branching. Aerial mycelia, when present, were scant and poorly developed without any spore bearing structures. On some media, small spherical vesicles (4~8 μm diameter) were found along the vegetative mycelia. On yeast malt extract agar these vesicles appeared to be pigmented.

Miscellaneous Physiological Reactions

The culture did not produce H_2S in peptone-iron agar. Melanoid pigments were not formed in peptone-iron agar or tryptone-yeast extract broth. On yeast extract malt extract agar the culture produced a yellow diffusible pigment. Slight hydrolysis of starch was also observed. The carbon source utilization pattern was as follows: moderate utilization of D-arabinose, L-arabinose, D-fructose, D-glucose, inositol, D-maltose, D-mannitol, D-mannose, D-raffinose, L-rhamnose and D-xylose; poor utilization of α-D-lactose, β-D-lactose, and sucrose.

Characterization of MA7095

Our continued search for novel inhibitors of HIV-1 reverse transcriptase resulted in the detection of MA7095 that produced two novel chromopeptolides. In an attempt to identify MA7095 various chemotaxonomic characteristics were examained. MA7095 has a Type III cell wall and a Type C whole cell sugar pattern. Member species of eight genera of the *Actinomycetales* exhibit these properties and can be further distinguished on the basis of morphological and additional chemotaxonomic characteristics such as fatty acid composition. Morphological studies of MA7095 failed to reveal spore-bearing structures under any of the growth conditions employed. Members of the genera *Actinomadura*, *Microbispora*, *Microtetraspora*, *Geodermatophilus*, *Actinosynnema*, *Nocardiopsis*, *Saccharothrix* and *Streptoalloteichus* all produce spore bearing structures under the conditions employed in these studies.

Fatty acid analysis can also be utilized to characterize actinomycetes in the absence of spore-bearing structures. To determine whether MA7095 was a non-sporulating

Table 1. Major whole cell fatty acids found in MA7095.

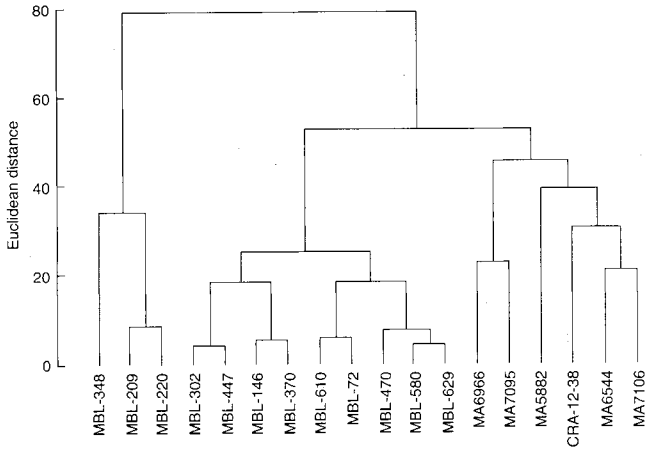
Fatty acid	%*
14:0 iso	1.31
15:0 iso	4.16
15:0	1.60
16:1 iso G	6.15
16:0 iso	42.40
16:0	1.04
unk 16.048	2.40
16:0 10 CH_3	1.75
17:0 iso	1.5
17:1 cis 9	3.50
16:0 iso 2OH	9.45
17:0 10 CH_3	19.18
18:1 cis 9	0.80
18:0	2.49

* Percentage of total fatty acids.

Table 2. Cultural characteristics of actinomycete sp. MA7095.

Medium	Amount of growth	Aerial mycelium	Soluble pigments	Substrate mycelium
Yeast malt extract agar	Excellent	None	Orange	Grayish, red brown (46 ge.r.br) Spherical, pigmented vesicles present
Sucrose-nitrate yeast extract agar	Very good	Sparse without any spore bearing structures	None	Slight reddish orange (35 s.rO)
Inorganic salts-starch agar	Fair	Sparse without any spore bearing structures	None	Brownish orange (54 brO). Small, nonpigmented vesicles
Oatmeal agar	Excellent	None	None	Brownish orange (54 brO). Vesicles rare.
Tap water agar	Fair	None	None	Slight yellow pink (26 S. yPink)
Czapek agar	Poor	None	None	Light yellow pink (28 l.yPink)
Peptone iron agar	Moderate (H_2S negative)			

Fig. 1. Dendogram of hierarchial analysis based on fatty acid composition.



member of a validly named genus, it was compared by this method to 1600 other strains of actinomycetes using tree-based models for classification and identification. These strains included approximately 600 taxonomic reference strains (500 streptomycetes and 100 plus members of 24 other genera-suprageneric groups) and 1000 wild-type strains from temperate and tropical terrestrial habitats and temperate marine habitats.

Following classification, MA7095 was placed into terminal nodes that contained 18 other strains. The relationship among these isolates was then further explored by hierarchical cluster analysis. Euclidean distance was used as the metric and the complete linkage method was used to construct the attached dendrogram (see Fig. 1). Thirteen of the strains were nocardioform isolates recovered from cold-water marine habitats off the coast of Cape Cod, MA (12/13 strains) or from tropical soils (1/13). The remaining four strains included the two samples of *Actinosynnema pretiosum* (MA6544 and MA7106), and two patent strains of *Amycoloata orientalis* (MA6966) and *Sebekia benihana* (MA5882/NRRL11111, *species incertia sedis*, U.S. Patent 4,128,563). Although the nearest neighbor to MA7095 is the *Amycolata* strain, it is unlikely that MA7095 belongs to this genus. *Amycolata* spp. have a type IV cell wall and arabinose and galactose as the whole cell sugars. In addition, the characteristics zig-zag growth of the vegetative mycelium was lacking in MA7095. It is also unlikely that MA7095 is a strain of *Actinosynnema pretiosum* since the culture does not produce synnemata, a characteristic trait of that genus. It is possible that MA7095 is aligned with *Sebekia benihana*, however, there are sufficient differences in micromorphology, cell chemistry and carbon source utilization to easily distinguish between these cultures. Based upon habitat and geographic factors, it is equally unlikely that MA7095 is closely related to the wild-type marine isolates or terrestrial isolates. These findings suggest that MA7095 may be an isolate of a previously undescribed species of actinomycete. Placement into the appropriate suprageneric grouping will require additional studies, including phylogentic analysis.

Further attempts to identify MA7095 involved the use of tDNA-PCR (polymerase chain reaction) fingerprinting[23]. The results (not shown) indicate that the band pattern from MA7095 did not match band patterns of isolates from over 30 different genera of actinomycetes. These data, taken together with the fatty acid analysis, clearly point to the uniqueness of MA7095.

Fig. 2. Structures of quinoxapeptin A and quinoxapeptin B.

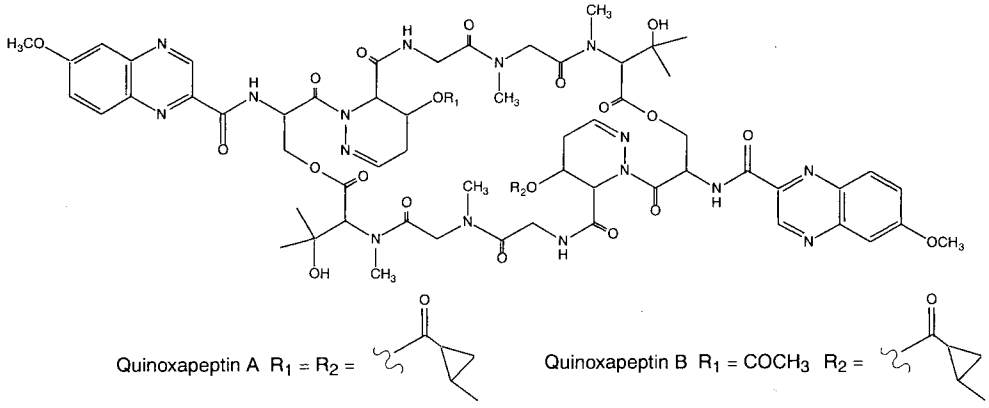
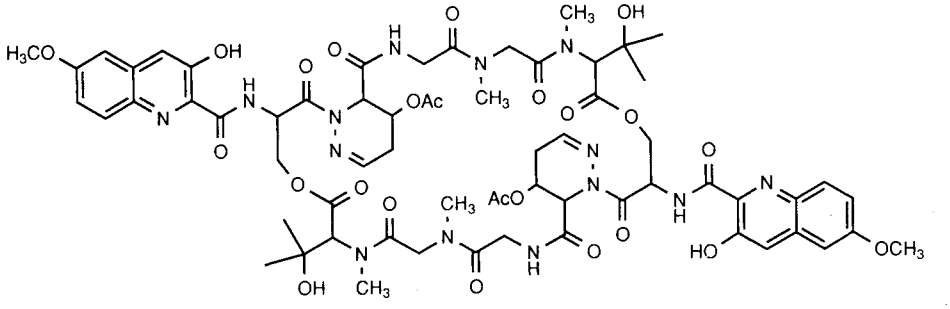


Fig. 3. Structure of luzopeptin A.



Inhibitor Isolation

The structures of quinoxapeptin A and B are presented in Fig. 2 while the structure of luzopeptin A is presented in Fig. 3. Overall, there is a great deal of similarity between luzopeptin and the quinoxapeptins that comprise the basis of this report. Structurally, the quinoxapeptins contain the same peptide backbone but differ in the attached chromphores and the acyl substituent appended to the tetrahydropyrazidine-3-carboxylic residue. The isolation and structure determination of quinoxapeptin A and B are presented in a separate communication[10].

Inhibitor Characterization

Quinoxapeptin A and B were evaluated for inhibition of HIV-1 and HIV-2 RT and to assess their selectivity towards reverse transcriptase (Table 3). Quinoxapeptin A inhibited HIV-1 and HIV-2 RT with IC_{50} values (the concentration of compound that elicits 50% inhibition) of 4 and 40 nM, respectively. Quinoxapeptin B was less potent and inhibited HIV-1 and HIV-2 RT with IC_{50} values of 10 and 100 nM, respectively while luzopeptin A inhibited HIV-1 and HIV-2 RT with IC_{50} values of 7 and 68 nM, respectively. Furthermore, Quinoxapeptin A and B were not time-dependent inhibitors of either HIV-1 or HIV-2 RT.

Table 3. Inhibition of HIV-1 and HIV-2 reverse transcriptase activity by chromodepsipeptides.

Inhibitor	IC_{50} (nM)	
	HIV-1 RT	HIV-2 RT
Quinoxapeptin A	4	40
Quinoxapeptin B	10	100
Luzopeptin A	7	68

HIV-1 and HIV-2 RT assays were performed as described[20,21]. Results are the mean of three separate experiments done in quadruplicate.

We have previously reported that rubromycin, another natural product inhibitor of HIV-1 RT, inhibited HIV-1 RT by competing with the template-primer substrate[21]. To determine the mechanism of inhibition of quinoxapeptin A a more detailed kinetic analysis was performed. The results of these studies (Figs. 4, 5 and Table 4) indicate that quinoxapeptin A was a non-competitive inhibitor of both HIV-1 and HIV-2 RT with *Ki* values of 18 and 22 nM, respectively. The almost identical

Fig. 4. Inhibition profile of quinoxapeptin A against HIV-1 reverse transcriptase.

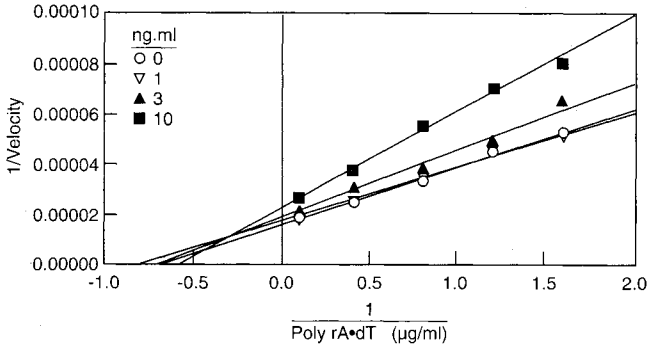


Fig. 5. Inhibition profile of quinoxapeptin A against HIV-2 reverse transcriptase.

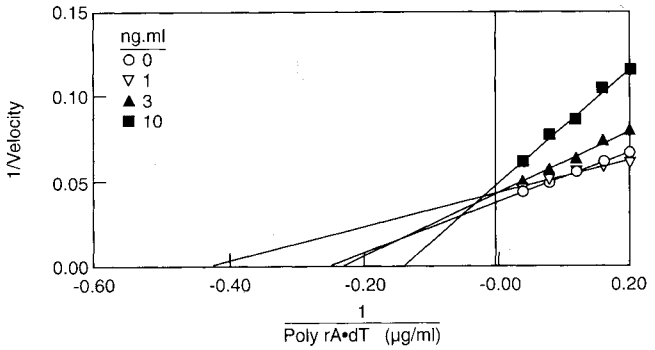


Table 4. Inhibition patterns and constants for quinoxapeptin A.

Enzyme	Quinoxapeptin A	
	With respect to r(A)d(T)	
	Type of inhibition	*Ki* (nM)
HIV-1 RT	Noncompetitive	17.6±1.6
HIV-2 RT	Noncompetitive	22.4±7.5

Kinetic analyses of HIV-1 and HIV-2 RT were performed as described[20]. Results are from one experiment (performed in quadruplicate) and are representative of two other experiments. Kinetic data were analyzed using a computer program (Kcat, Princeton, New Jersey) to obtain the kinetic constants.

Table 5. Inhibition of HIV-1 RT mutants and DNA polymerases by quinoxapeptin A.

Enzyme	IC_{50} (nM)
HIV-1 RT mutants	
Single mutant-Y181C	12
Single mutant-K103N	8
Double mutant-Y181C/K103N	6
DNA polymerases	
α	2563
β	615
γ	1798
δ	494

Enzyme assays were perfomed as described[20,21]. The mutant enzymes were prepared, expressed and isolated as described[22].

sensitivity of both enzymes to quinoxapeptin A suggests that the active sites and template-primer binding domains on both enzymes may be equivalent. Quinoxapeptin A presumably inhibits both enzymes by intercalating with the template-primer forming an adduct that is no longer recognized by the viral enzymes. A recent report[24] indicated that acridine-like compounds inhibited HIV-1 RT in a similar manner. They also reported that double-reciprocal plots of kinetic data appeared to be mixed/non-competitive in nature.

During the development of several non-nucleoside inhibitors of HIV-1 RT it became clear that the HIV-1 virus was developing resistance to the various compounds. In an attempt to understand this phenomenon, virus was isolated from infected individuals and the viral genome was sequenced. Several mutations were found in the RT coding region including changes in position 181 where a tyrosine was changed to a cysteine (Y181C, single letter amino acid code). Another isolate of the virus contained a substitution of asparagine for lysine at position 103 (K103N). A third variant included the double mutation (Y181C/K103N). The mutations map to a region outside the primer-template and dNTP binding sites[22].

We examined whether quinoxapeptin A was active against these three mutant forms of HIV-1 RT and the results are presented in Table 5. Quinoxapeptin A exhibits IC_{50} values of 12, 8 and 6 nM against these three variant enzymes, respectively. This is only two-three fold less active than against the wild-type enzyme (IC_{50} = 4 nM, Table 3). The data suggest that the mode of inhibition of the mutant enzymes by quinoxapeptin A is probably different from that of the non-nucleoside inhibitors. This is consistent with the observed inhibition kinetics that indicate that that quinoxapeptin A may be interacting with the template-primer binding site.

To further examine the specificity of quinoxapeptin A it was tested against several mammalian DNA polymerases and the results are also presented in Table 5. Quinoxapeptin A inhibited DNA polymerase α, β, γ and δ with IC_{50} values of 2563, 615, 1798 and 494 nM, respectively, once again confirming the exquisite

Table 6. Reversibility of quinoxapeptin A against HIV-1 and HIV-2 reverse transcriptases.

Conditions	pmol/mg/hour	Percent inhibition
HIV-1 RT		
I. Initial incubation and no gel-filtration		
1. HIV-1 RT + 5% DMSO	27262	
2. HIV-1 RT + 20.3 nM quinoxapeptin A	11570	57
II. After gel-filtration		
3. HIV-1 RT + 5% DMSO + gel-filtration + 5% DMSO in assay	39857	
4. HIV-1 RT + 5% DMSO + gel-filtration + 20.3 nM quinoxapeptin A in assay	14134	65
5. HIV-1 RT + 20.3 nM quinoxapeptin A + gel-filtration + 5% DMSO in assay	40051	0
6. HIV-1 RT + 20.3 nM quinoxapeptin A + gel-filtration + 20.3 nM quinoxapeptin A in assay	10887	65
HIV-2 RT	pmol/unit/hour	
I. Initial incubation and no gel-filtration		
7. HIV-2 RT + 5% DMSO	13.37	
8. HIV-2 RT + 203 nM quinoxapeptin A	8.7	35
II. After gel-filtration		
9. HIV-2 RT + 5% DMSO + gel-filtration + 5% DMSO in assay	24.74	
10. HIV-2 RT + 5% DMSO + gel-filtration + 203 nM quinoxapeptin A in assay	7.52	70
11. HIV-2 RT + 203 nM quinoxapeptin A + gel-filtration + 5% DMSO in assay	19.89	20
12. HIV-2 RT + 203 nM quinoxapeptin A + gel-filtration + 203 nM quinoxapeptin A in assay	6.75	73

Tubes containing human HIV-1 (2 nM) or HIV-2 RT (16 units) were incubated with either: (i) DMSO, (ii) 20.3 mM quinoxapeptin A (for HIV-1 RT) or (iii) 203 nM quinoxapeptin A (for HIV-2 RT) for 25 minutes at 31°C. Aliquots (10 μl) were removed and assayed directly (see Condition I of the table). The remainder of the three incubations were gel-filtered through 2.2 ml Sephadex G-25 columns equilibrated with 80 mM Tris, pH 8.2, 8 mM dithiothreitol, 0.14% bovine serum albumin abd 0.01% Triton X-100. The column eluates (25 μl), in column equilibration buffer, were tested for HIV-1 or HIV-2 RT activity as previously described[21]. DMSO or quinoxapeptin A was added back to the assay tubes as shown above. The data represent mean of quadruplicates for each condition from one experiment.

specificity of quinoxapeptin A for HIV-1 and HIV-2 RT.

To examine whether quinoxapeptin A was a reversible inhibitor of HIV-1 and HIV-2 RT the experiment presented in Table 6 was performed. HIV-1 or HIV-2RT was pre-incubated (separately) with either quinoxapeptin A or DMSO. Aliquots were removed for assay, while the remainder of the samples were passed through columns of Sephadex G-25. Testing of each sample before gel-filtration indicated that the HIV-1 and HIV-2 RT activity was inhibited by quinoxapeptin A (rows 2 and 8). Following gel-filtration, the control sample was inhibited by re-addition of quinoxapeptin A (rows 4 and 10). The HIV-1 and HIV-2 RT activity of the samples pre-incubated with quinoxapeptin A following gel-filtration was similar to the control sample suggesting that the inhibitors had dissociated from the enzyme (rows 5 and 11). Re-addition of quinoxapeptin A to these samples reduced HIV-1 and HIV-2 RT activity to the same degree as the control (rows 6 and 12). These data indicate that quinoxapeptin A is a reversible inhibitor of both HIV-1 and HIV-2 RT.

Discussion

The quinoxapeptins isolated from MA7095, while being novel, are structurally related to the luzopeptins described earlier[3~6]. More recently, the isolation of sandriamycin, another chromodepsipeptide related to the luzopeptins has been described[7,8]. These compounds were both antibiotics and antitumorigenic[3,7]. However, there was no mention of whether these compounds were effective as antivirals. In contrast to the previous data[3,7], luzopeptin A and quinoxapeptin A were cytotoxic in a viral spread assay for the HIV-1 virus (results not shown). Reasons for this are unclear but may include that the cell line used in these earlier studies was less sensitive to these compounds.

Recently, the effect of one hundred and fifty-six compounds isolated from natural product sources that were tested against HIV-1 and HIV-2 RT was described[25]. The most potent of these included fagaronine chloride and nitidine chloride that were equally active against both HIV-1 and HIV-2 RT (IC_{50} values of 20 μM[25]). This is in contrast to quinoxapeptin A which is almost 5,000 times more active than either of these compounds against HIV-1 RT (Table 4). These authors also speculated that these compounds were probably disrupting nucleic acid template functions[25].

The development of resistance to therapeutic agents by the HIV-1 virus is probably a function of viral mutation and the lack of fidelity of reverse transcriptase

activities[26~28]. The discovery of quinoxapeptin A and B from microbial sources that are specific inhibitors of HIV-1 and HIV-2 RT as well as being active against three variants of HIV-1 RT is encouraging in that it validates the concept of screening natural product extracts for unique secondary metabolites that may be used as potential drug development candidates.

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