Differential Contribution of Metal Complexation and Dimerization to the Chemotherapeutic Potential of Bicyclen- Zn^{II} Complex against Human Immunodeficiency Virus

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1,1'-(*m*-Xylenediyl)-bis(1,4,7,10-tetraazacyclododecane)- Zn^{II} complex (*m*-xylenediyl-bicyclen- Zn^{II}), a potent inhibitor of human immunodeficiency virus (HIV), was obtained from cyclen by a combination of dimerization and metal complexation. The ratio of median cytotoxic concentration against host cells (CC_{50}) and median effective concentration against HIV cytopathogenicity (EC_{50}), referred to as the selectivity index (*SI*), was regarded to be a measure of anti-HIV activity. These two chemical modifications contributed to a potent, *in vitro* anti-HIV activity of *m*-xylenediyl-bicyclen- Zn^{II} by respectively increasing the CC_{50} and decreasing the EC_{50} in comparison with those of cyclen.

Key words anti-HIV activity; macrocyclic polyamine; cyclen; metal complexation; dimerization

Saturated macrocyclic polyamines such as a 12-membered tetraamine, cyclen (1,4,7,10-tetraazacyclododecane), and a 14-membered tetraamine, cyclam (1,4,8,11-tetraazacyclotetradecane), are known to be a group of compounds providing promising agents to combat human immunodeficiency virus (HIV). In 1992, De Clercq *et al.* reported that a series of *N,N'*-bridged bicyclams, which contain two cyclam subunits bridged by an aliphatic group [e.g., JM2763, 1,1'-(1,3-propylene)-bicyclam·8HCl·2H₂O], are potent and selective inhibitors of HIV type 1 (HIV-1) and type 2 (HIV-2), presumably due to interference with the uncoating process of HIV replication.³⁾ The authors subsequently discovered novel *C,C'*-bridged bicyclams [e.g., IPS31, 6,6'-(1,3-propylene)-bicyclam·8HCl·0.5H₂O] with potent anti-HIV activity.⁴⁾ The delayed toxicity seemed to be to their disadvantage as far as application to anti-HIV chemotherapeutics was concerned.⁵⁾ However, this could be overcome by complexing them with divalent transition metal ions such as Zn^{II} , Cu^{II} and Ni^{II} ,^{4,5)} the complexes are extremely stable under physiological conditions.

Recently, De Clercq *et al.*⁶⁾ showed that bicyclams in which the cyclam subunits are joined by aromatic linkers [e.g., JM3100, 1,1'-(*p*-xylenediyl)-bicyclam·8HCl·2H₂O] exhibited much higher potency than bicyclams containing aliphatic linkers.

In contrast, cyclen failed to show anti-HIV activity below its CC_{50} of 20 μM .⁵⁾ Although as toxic as cyclen, bicyclens such as 1,1'-(*m*- or *p*-xylenediyl)-bicyclen·8HBr suppressed the cytopathogenicity of both HIV-1 and -2. They were far inferior to bicyclams in terms of selectivity index (*SI*) mainly due to their lower CC_{50} s.⁷⁾ When complexed with divalent metal ions, the toxicity of cyclen was reduced markedly by a factor of 400- to 500-fold, resulting in unmasking of the anti-HIV activity.⁵⁾ Based on these findings, bicyclen-metal complexes containing aromatic linkers are expected to possess marked anti-HIV potentials.

In this paper, a comparative study of the contributions

of dimerization and metal-complexation to the anti-HIV activity of bicyclen-metal complexes has been made.

MATERIALS AND METHODS

Cyclen·4HCl (IPS1) was obtained commercially. Cyclen- Zn^{II} ·(ClO_4)₂·H₂O (IPS4) was prepared by a previous method.⁵⁾ The synthesis of 1,1'-(*m*-xylenediyl)-bicyclen·8HBr·3H₂O (IPS150),^{8a)} 1,1'-(*m*-xylenediyl)-bicyclen- Zn^{II} ·(ClO_4)₃·OH·H₂O (IPS151),^{8a)} 1,1'-(*p*-xylenediyl)-bicyclen·8HBr·4H₂O (IPS152)^{8b)} and 1,1'-(*p*-xylenediyl)-bicyclen- Zn^{II} ·(ClO_4)₄·4H₂O (IPS153)^{8b)} is to be published elsewhere.

Fig. 1. Structures of Cyclens and Bicyclens

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Cells and Viruses MT-4 cells, MOLT-4 clone C10 cells established in our laboratory⁵⁾ and MOLT-4 cells chronically infected with the HIV strains (MOLT-4/HIV-1_{HTLV-IIIb}, MOLT-4/HIV-1_{RF}, MOLT-4/HIV-1_{MN}, MOLT-4/HIV-1_{SF-2H}, MOLT-4/HIV-2_{ROD} and MOLT-4/HIV-2_{GH-1}), were maintained in RPMI-1640 medium supplemented with 10% fetal calf serum; penicillin, 100 units/ml and streptomycin, 100 µg/ml were used. Virus stocks of HIV-1_{HTLV-IIIb}, HIV-1_{RF}, HIV-1_{MN} and HIV-2_{ROD} were obtained from the culture supernatants of MOLT-4/HIV-1_{HTLV-IIIb}, MOLT-4/HIV-1_{RF}, MOLT-4/HIV-1_{MN} and MOLT-4/HIV-2_{ROD}, respectively. The titers of the virus preparations determined in MT-4 cells 4 d after infection were 4.5×10^4 , 6.8×10^5 , 2.6×10^5 and 7.5×10^5 median tissue culture inhibitory concentration (TCID₅₀)/ml, respectively.

Anti-HIV Assay in HIV-Induced Cytopathogenic Effect (CPE) The measurement of the antiviral activity of a compound against HIV-1_{HTLV-IIIb}, HIV-1_{RF}, HIV-1_{MN} and HIV-2_{ROD} was based on the inhibition of virus-induced CPE in MT-4 cells. Briefly, MT-4 cells were infected with HIV at a multiplicity of infections (moi) of 0.01 for 1 h at 37°C. Cells were incubated with individual test compounds in 96-well microtiter trays for 5 d at 37°C in a total volume of 200 µl. Cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) metabolic assay.⁹⁾

The 50% effective concentration (EC₅₀) was defined as the concentration at which CPE was inhibited by 50%. The 50% cytotoxic concentration (CC₅₀) of each compound was defined as the concentration at which the cell viability of mock-infected MT-4 cells, measured in parallel with the determination of EC₅₀, was reduced by half.

Although no infectious particles were released by MOLT-4/HIV-1_{SF-2H} cells into medium, cocultivation of MT-4 and MOLT-4/HIV-1_{SF-2H} cells caused marked CPE in MT-4 cells due to cell-to-cell virus transmission. MOLT-4 or MOLT-4/HIV-1_{SF-2H} cells were cocultured with MT-4 cells at a ratio of 1:20 in the presence of test compound for 5 d at 37°C. The CC₅₀ and EC₅₀ values were determined by the cell viabilities of MT-4 cells cocultured with MOLT-4 and MOLT-4/HIV-1_{SF-2} cells,

respectively. The details of this cell-to-cell infection inhibition assay have been published elsewhere.¹⁰⁾

Syncytium Formation Inhibition Assay MOLT-4 clone C10 and MOLT-4/HIV cells were cocultured at a ratio of 2:1 or individually at a final cell density of 2.5×10^5 cells/ml in the presence of various concentrations of test compound at 37°C for 20 h. Control wells contained either MOLT-4 cells, MOLT-4/HIV cells or their 2:1 mixture in the absence of test compounds. The number of cells was determined in a Coulter counter, Model DN. The fusion index (FI) was defined as follows¹¹⁾: $FI = [(cell\ number\ in\ MOLT-4\ well) \times 2 + (cell\ number\ in\ MOLT-4/HIV\ well)] / [(cell\ number\ in\ mixed-culture\ well) \times 3] - 1.0$.

The FIs in control drug-free cultures ranged from 0.5 to 1.2. The percentage reduction in $FI = (1 - FI_T/FI_C) \times 100$, where FI_T is FI of the test compound and FI_C is that of the control. The 50% inhibitory concentration (IC₅₀) was defined as the concentration at which FI was reduced by half.

RESULTS

Inhibition of HIV-Induced CPE The bicyclens, IPS150 through IPS153, were studied for their inhibitory activity against CPE in MT-4 cells induced by HIV-1_{HTLV-IIIb}, HIV-1_{RF}, HIV-1_{MN}, HIV-1_{SF-2H} and HIV-2_{ROD}. The results are summarized in Table 1 as well as the data for the monomeric cyclens, IPS1 and IPS4, against HIV-1_{HTLV-IIIb}. The role of metal complexation was deduced by pairing the data in the following combinations: IPS1 vs. IPS4, IPS150 vs. IPS151 and IPS152 vs. IPS153. Marked enhancement in CC₅₀ resulted from metal complexation irrespective of the back-bone structure (cyclen, *m*-xylenediyl-bicyclen or *p*-xylenediyl-bicyclen), while no effect on EC₅₀ was noticeable. On going from IPS1 to IPS150 and IPS152, the shift in CC₅₀ was trivial, while the EC₅₀ was measurable only in the case of bicyclens. In the case of IPS1, EC₅₀ could not be measured over a range of subtoxic concentrations. In an analogy to the relationship between the EC₅₀s of IPS150 and IPS151, and IPS152 and IPS153, a EC₅₀ of IPS1 was estimated to be

Table 1. Inhibition of HIV-Induced Cytopathogenicity by Cyclens and Bicyclens

Compound (Metal)	Inhibition assay in HIV-induced CPE														
	HIV-1 _{HTLV-IIIb}			HIV-1 _{RF}			HIV-1 _{MN}			HIV-1 _{SF-2H} ^{a)}			HIV-2 _{ROD}		
	CC ₅₀ (µM)	EC ₅₀ (µM)	SI ^{b)}	CC ₅₀ (µM)	EC ₅₀ (µM)	SI ^{b)}	CC ₅₀ (µM)	EC ₅₀ (µM)	SI ^{b)}	CC ₅₀ (µM)	EC ₅₀ (µM)	SI ^{b)}	CC ₅₀ (µM)	EC ₅₀ (µM)	SI ^{b)}
Cyclen															
IPS1	20	NA ^{c)}	—												
IPS4 (Zn ^{II})	6300	520	12												
<i>m</i> -Xylenediyl-bicyclen															
IPS150	6.2	0.088	70	6.2	0.28	27	6.4	0.10	64	17	0.21	81	2.3	0.50	4.6
IPS151 (Zn ^{II})	>1100	0.049	>22000	>1100	0.26	>4200	>1100	0.17	>6500	>1100	0.19	>5800	>1100	0.93	>1200
<i>p</i> -Xylenediyl-bicyclen															
IPS152	13	0.26	50	13	1.02	13	13	0.38	34	18	0.88	20	10	3.9	2.6
IPS153 (Zn ^{II})	750	0.14	5400	820	0.84	980	870	0.24	3600	800	0.67	1200	720	11	65

Data represents mean values for two experiments. Cell viability was measured by the MTT metabolic assay 5 d after virus infection. a) MOLT-4/HIV-1_{SF-2H} cells were used instead of free virions. b) Selectivity index corresponding to the ratio of CC₅₀/EC₅₀. c) Not available because CPE inhibition was less than 50% at non-toxic concentrations.

Table 2. Inhibition of HIV-Dependent Syncytium Formation by Bicyclens

Compound (Metal)	Syncytium formation inhibition assay, IC ₅₀ (μ M)		
	HIV-1 _{HTLV-IIIb}	HIV-1 _{SF-2H}	HIV-2 _{GH-1}
<i>m</i> -Xylenediyl-bicyclen			
IPS150	0.85	0.15	0.021
IPS151 (Zn ^{II})	0.84	0.11	0.026
<i>p</i> -Xylenediyl-bicyclen			
IPS152	> 5.0	0.58	0.077
IPS153 (Zn ^{II})	2.2	0.29	0.076

HIV-infected MOLT-4 cells (MOLT-4/HIV-1_{HTLV-IIIb}, MOLT-4/HIV-1_{SF-2H}, MOLT-4/HIV-2_{GH-1}) and uninfected MOLT-4 clone C10 cells were cultured individually or in combination at a ratio of 1:2 for 20 h in the presence of various inhibitors. Each culture was established with 3.0×10^5 cells in a total volume of 1.2 ml.

approximately 500 μ M for HIV-1_{HTLV-IIIb}, which might be masked by a far lower CC₅₀ (20 μ M). On going from IPS4 to IPS151 and IPS153, a marked effect of dimerization was apparent only in the EC₅₀. Thus, dimerization seemingly potentiates the anti-HIV activity of cyclen monomers in the presence or absence of metal ions.

Common strain specificity was shared by all four bicyclens when analyzed in terms of their EC₅₀s. The susceptibility of various HIV strains to bicyclens is in the following order; HIV-1_{HTLV-IIIb}, HIV-1_{MN}, HIV-1_{RF} and HIV-2_{ROD}. For all strains, *m*-xylenediyl-bicyclens were more potent than *p*-xylenediyl-bicyclens and the relative susceptibility to *m*-xylenediyl-bicyclens over *p*-xylenediyl-bicyclens was highest in the case of HIV-2_{ROD}.

Effect on Syncytium Formation The bicyclens and their Zn^{II} complexes were added to a mixed culture of uninfected and HIV-infected MOLT-4 cells, to evaluate their inhibitory effects on syncytium formation in terms of IC₅₀. The strains tested were HIV-1_{HTLV-IIIb}, HIV-1_{SF-2H} and HIV-2_{GH-1} (Table 2). Among them, HIV-2_{GH-1} is the most susceptible strain with an IC₅₀ of 0.02 μ M (*m*-xylenediyl-bicyclens) and 0.08 μ M (*p*-xylenediyl-bicyclens). The susceptibility of syncytium formation induced by HIV-1_{HTLV-IIIb} and HIV-1_{SF-2H} was inversely correlated with that of CPE. As in the case of CPE, syncytium formation was inhibited by *m*- and *p*-xylenediyl-bicyclens to different extents, independently of metal complexation.

DISCUSSION

Cyclen is highly toxic when compared with cyclam, although its toxicity is easily reversed by complexation with divalent transition metal ions.⁵⁾ As shown in Table 1, the dimerization resulted in a marked decline in EC₅₀ (IPS4 vs. IPS151 or IPS153) which was scarcely affected by metal complexation (IPS150 vs. IPS151, IPS152 vs. IPS153). However, the toxicity of cyclen was not affected by dimerization because the CC₅₀s of IPS1, IPS150 and IPS152 were in the same range (Table 1). This was also true for the Zn^{II} complexes. In combination, these two chemical modifications enhanced the SI (CC₅₀/EC₅₀) synergistically. In contrast to the case of bicyclams,⁷⁾ bicyclens were more potent in the *m*-configuration in the xylenediyl linker moiety than in the *p*-configuration.

In the *in vitro* assay system employed in this study, the

reciprocals of EC₅₀ and CC₅₀ represent the magnitudes of anti-HIV activity (effect) and toxicity (side-effect), respectively. Based on their independent response to dimerization and metal complexation and the fact that EC₅₀ is virus-dependent and CC₅₀ is host cell-dependent, the effects and side-effects are probably triggered by interaction of cyclens/bicyclens with different target molecules (tentatively named T_e and T_s, respectively).

The interaction of cyclen with T_e leads to inhibition of HIV replication. Dimerization might fortify this interaction, while metal complexation has no effect. In contrast, host cell integrities are severely damaged by interaction between cyclens/bicyclens and T_s, which could be reversed significantly by metal complexation.

Inhibition of syncytium formation, which was susceptible to inhibitors of virus adsorption and/or virus-cell membrane fusion, was studied to identify the site of action of bicyclens. In 1992, for the first time, De Clercq *et al.*³⁾ reported inhibition of syncytium formation by bicyclams at far higher concentrations than those at which they interfered with CPE. We have observed marked inhibition of syncytium formation by our original bicyclams in a system consisting of MOLT-4 cells uninfected and infected with HIV-2_{GH-1}.⁴⁾ The IC₅₀s of bicyclams against HIV-2_{GH-1}-dependent syncytium formation were comparable with or even lower than the EC₅₀s against the CPE of other strains. The same was true for bicyclens tested in the present study, as shown in Tables 1 and 2. The EC₅₀ and IC₅₀ are proportional to each other for individual HIV strains, suggesting that bicyclens may interfere with virus replication at some early stage such as virus adsorption and membrane fusion.

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