Biochemical Pharmacology, Vol. 44, No. 8, pp. 1675–1679, 1992.
Printed in Great Britain.

0006-2952/92 $5.00 + 0.00

SHORT COMMUNICATIONS

Anti-human immunodeficiency virus effects of cationic metalloporphyrin–ellipticine complexes

(*Received* 10 *June* 1992; *accepted* 23 *July* 1992)

Abstract—A series of cationic metalloporphyrin–ellipticine complexes were found to inhibit the cytopathicity of human immunodeficiency virus type 1 (HIV-1) and simian immunodeficiency virus in MT-4 cells at concentrations ranging from 1.4 to 17 μg/mL, i.e. at a concentration that was 2.5–30-fold below the cytotoxicity threshold. These compounds were also found to inhibit syncytium formation between persistently HIV-1-infected HUT-78 and uninfected Molt/4 cells, to interfere with HIV-1 binding to the cells, and to suppress HIV-1-associated reverse transcriptase activity.

The development of new antiviral drugs for AIDS treatment has been the subject of intensive research since human immunodeficiency virus (HIV*) was identified as the causative agent of the disease [1–4]. Among the possible strategies that could be considered figure the antisense oligonucleotides which can inhibit HIV replication at different stages, i.e. transcription, translation, integration of viral DNA into the host genome [5–7]. In the field of oligonucleotides, research is in progress to achieve optimal nuclease resistance and cellular uptake and to improve the efficiency of oligonucleotides by coupling reactive groups to create irreversible damages on the targeted nucleic acid sequence (for the attachment of DNA cleaving agents to oligonucleotides, see Ref. 8). However, the currently used DNA cleaving agents in cell culture systems have so far not been demonstrated to be effective, because of their difficulty in entering the cells and/or rapid metal exchange inside the cells.

During our studies on bleomycin models [9, 10], we found that the tris-methylpyridinium-porphyrin moiety can be considered as a suitable chelating agent to be attached to a vector to help in the recognition of DNA sequences (for a recent review article on sequence-selective DNA recognition by various chemicals, see Ref. 11). This ligand has (i) a strong affinity for DNA ($K_{aff} = 10^4$–$10^5\ M^{-1}$) [10, 12], (ii) a high nuclease activity and (iii) a metal-dependent cytotoxic activity [9, 10]. In addition, manganese porphyrins are not readily demetallated *in vivo* [13]. High affinity for DNA and absence of *in vivo* demetallation or metal exchange are necessary for hybrid metalloporphyrin–oligonucleotide molecules to be possibly useful as "DNA or RNA-directed chemotherapeutic agents". Previous studies have been focussed on the oxidative degradation of DNA by the parent metalloporphyrin complex, *meso*-tetrakis(4-*N*-methylpyridiniumyl)porphyrinatomanganese-(III) pentaacetate [14, 15]. During the course of our work on the preparation of cationic metalloporphyrin–oligonucleotides, we wanted to investigate the possible antiviral activity of the tris(methylpyridiniumyl)porphyrinatomanganese moiety.

Materials and Methods

Chemicals. The water-soluble hybrid molecules (Fig. 1) were synthesized as described previously [10] by coupling a functionalized derivative of 9-methoxyellipticine, a known intercalating agent [16], with a tris(methylpyridiniumyl)porphyrin moiety [17]. Relative molecular masses are as follows: **1-Mn**, $M_r = 1385$; **2-Mn**, $M_r = 1470$; **3-Mn**, $M_r = 1444$; **3-Zn**, $M_r = 1396$; **4-Mn**, $M_r = 1458$. A manganese derivative based on the tris(methyl-pyridiniumyl)porphyrin skeleton was used to establish the biological activity of the metalloporphyrin unit of these hybrid molecules: the manganese complex of [5 - (4 - trimethylaniliniumyl) - 10,15,20 - tris(4 - *N*-methyl-pyridiniumyl)porphyrin pentaacetate, **5-Mn**, $M_r = 1069$ (see Fig. 2 for the structure and Ref. 17 for the synthesis). In all manganese complexes, an axial acetate ligand is assumed, but these water-soluble manganese complexes may have a water molecule in the axial position with an acetate as counter-anion.

Viruses. HIV type 1 (HIV-1) [strain HTV-III_B(LAI)] was originally obtained from the culture supernatant of the persistently HIV-1-infected H9 cell line (H9/HTLV-III_B) [18] and was kindly provided by R. C. Gallo and M. Popovic (National Cancer Institute, Bethesda, MD, U.S.A.). Virus stocks were prepared from the supernatants of HIV-1-infected MT-4 cells. HIV type 2 (HIV-2) (strain LAV-2, ROD) [19] was a gift from L. Montagnier (Institut Pasteur, Paris, France), and virus stocks were prepared from the supernatants of HIV-2-infected MT-4 cells. Simian immunodeficiency virus (SIV) (strain SIV_{MAC251}) was originally isolated by Daniel *et al.* [20] and was obtained from C. Bruck (Smith Kline-RIT, Rixensart, Belgium).

Antiretroviral activity assay procedure. The methodology of the anti-HIV assays has been described previously [21]. Briefly, MT-4 cells (4.5×10^5 cells/mL) were suspended in fresh culture medium and infected with HIV-1, HIV-2 or SIV at 100 $CCID_{50}$ (1 $CCID_{50}$ being the dose infective for 50% of the cell cultures) per mL of cell suspension. The infected cell suspension (100 μL) was then transferred to microtray wells, mixed with 100 μL of the appropriate dilutions of the test compounds. After 5 days, the number of viable cells was determined in a blood cell-counting chamber by Trypan blue staining. The 50% effective concentration (EC_{50}) and 50% cytotoxic concentration (CC_{50}) correspond to the compound concentrations required to reduce by 50% the number of viable cells in the virus-infected and mock-infected cell cultures, respectively.

Syncytium formation assay procedure. Persistently HIV-1- or HIV-2-infected HUT-78 cells (designated HUT-78/HIV-1 and HUT-78/HIV-2, respectively) were washed to remove free virus from the culture medium, and 5×10^4 cells (50 μL) were transferred to 96-well microtiter trays. To each well were then added 5×10^4 MOLT-4 (clone 8) cells (50 μL) and an appropriate concentration of the test

* Abbreviations: HIV, human immunodeficiency virus; HIV-1/2, HIV type 1/2; SIV, simian immunodeficiency virus; PBS, phosphate-buffered saline; FITC, fluorescein isothiocyanate; RT, reverse transcriptase; RaM, rabbit antimouse; IgG, immunoglobulin; mAb, monoclonal antibody; PE, phycoerythrine.

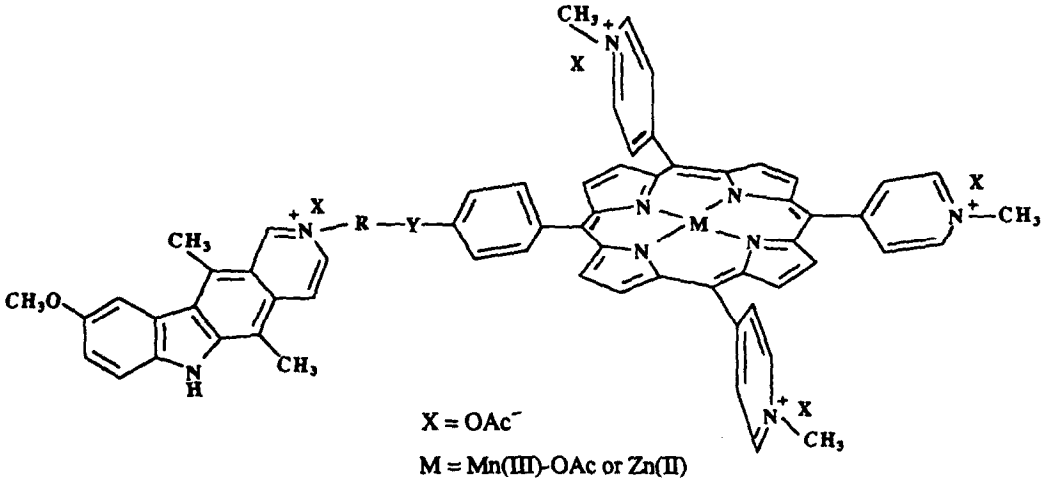

Fig. 1. Structures of metalloporphyrin–ellipticine complexes **1-Mn**, **2-Mn**, **3-Zn** and **4-Mn**.

compound (10 μL). The mixed cells were cultured at 37° in a CO_2-controlled atmosphere. After 16–20 hr, marked syncytium formation was noted, and syncytia were counted under the microscope.

Virus adsorption assay. The inhibitory effects of the test compounds on virus adsorption were measured by the indirect immunofluorescence-laser flow cytofluorographic method [23]. MT-4 cells were exposed briefly to HIV-1 virions in the presence or absence of the test compounds. The cells were incubated for 30 min at 37° and washed twice in phosphate-buffered saline (PBS). Then the cells were stained by subsequent treatment with a high-titer polyclonal antibody derived from a patient with AIDS-related complex [24] (diluted 1/500 in PBS) and fluorescein isothiocyanate (FITC)-conjugated $F(ab')_2$ fragments of rabbit anti-human immunoglobulin (IgG) antibody (diluted 1/30 in PBS). Finally, cells were fixed in 0.5% paraformaldehyde in PBS, and analysed by laser flow cytofluorography.

CD4 immunofluorescence assay. CD4 expression was determined by FACSTAR (Becton-Dickinson) analysis, as described previously [25]. MT-4 cells were briefly incubated in the absence or presence of serum with or without test compound. The cells were then stained with the monoclonal antibodies (mAbs) OKT4A-FITC (Ortho Diagnostics) or anti-leu3a-PE and Simultest immune monitoring kit control (FITC-labeled IgG_1 and PE-labeled IgG_2) (Becton-Dickinson), washed once in PBS, and fixed in 0.5% paraformaldehyde in PBS.

Glycoprotein gp120 immunofluorescence assay. HUT-78/HIV-1 cells (200,000 cells) in 100 μL of RPMI medium supplemented with 10% fetal calf serum were washed in

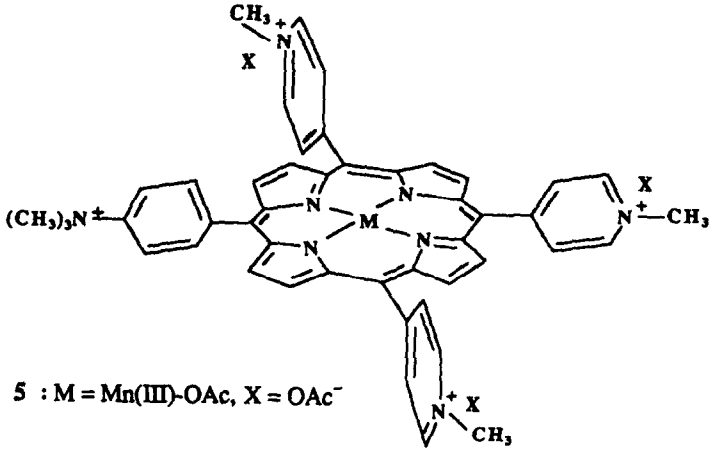

Fig. 2. Structure of [5-(trimethylaniliniumyl)-10,15,20-tris(4-*N*-methylpyridiniumyl)]porphyrinato-manganese(III) pentaacetate, **5-Mn**.

RPMI medium with 10% fetal calf serum, incubated with the compounds at various concentrations for 15–20 min, washed twice with RPMI medium to remove residual compound, stained with anti-gp120 mAb (9284, DuPont de Nemours, Brussels, Belgium) at 37°, washed twice in PBS, incubated with FITC-conjugated $F(ab')_2$ fragments of rabbit antimouse immunoglobulin antibody [RaM-IgG-$F(ab')_2$FITC] (Prosan, Ghent, Belgium) at 37°, washed twice in PBS, resuspended in 0.5 mL of 0.5% paraformaldehyde in PBS, and analysed by flow cytometry, as described previously [26]. The threshold of positivity for green fluorescence intensity was arbitrarily established on the basis of control samples of uninfected HUT-78 cells incubated with anti-gp120 mAb and RaM-IgG-$F(ab')_2$-FITC or of HIV-1-infected HUT-78 cells incubated solely with RaM-IgG-$F(ab')_2$-FITC.

Reverse transcriptase assay. The method for determination of the inhibitory effect of test compounds against HIV-1 reverse transcriptase (RT) has been described previously [27]. The reaction mixture (50 μL) contained 50 mM Tris–HCl (pH 7.8), 5 mM dithiothreitol, 300 mM glutathione, 500 μM EDTA, 150 mM KCl, 5 mM $MgCl_2$, 1.25 μg of bovine serum albumin, 2 μCi of [^{3}H]dTTP (sp. radioact., 24 Ci/mmol), 0.01 U of poly(A), oligo(dT)12–18, 0.03% Triton X-100, 10 μL of test compound solution (containing various concentrations of the compound) and 1 μL of recombinant HIV RT (p66) (generously supplied by P. J. Barr, Chiron). The reaction mixtures were incubated at 37° for 15 min, at which time 100 μL of calf thymus DNA (150 μg/mL), 2 mL of $Na_4P_2O_7$ (0.1 M in 1 M HCl) and 2 mL of trichloroacetic acid (10%, v/v) were added. The solutions were kept on ice for 30 min, after which the acid-insoluble material was washed and analysed for radioactivity.

Results

The test compounds were evaluated for their inhibitory effect on the cytopathicity of HIV-1, HIV-2 or SIV in MT-4 cells (Table 1). All compounds were found to inhibit HIV-1-induced cytopathicity at subtoxic concentrations, **3-Mn** being the most potent and most selective inhibitor. In contrast, none of the test compounds proved effective against HIV-2 at subtoxic concentrations, except for **5-Mn** which was effective at a concentration 1.5-fold lower than the cytotoxic concentration. Most test compounds were endowed with an anti-SIV effect at doses comparable with those required to inhibit HIV-1, except for **3-Mn** which was about 10-fold less effective against SIV than HIV-1 (Table 1).

Table 1. Antiretroviral and cytotoxic activity of metalloporphyrin–ellipticine complexes in human MT-4 lymphocyte cells

	EC_{50}* (μg/mL)			
Compound	HIV-1	HIV-2	SIV	CC_{50}† (μg/mL)
1-Mn	8.8	>20	11	29
2-Mn	9.5	>20	9.9	45
3-Mn	1.4	—	16	42
3-Zn	17	>20	>20	42
4-Mn	10	>20	12	44
5-Mn	11	42	≥20	62

* Fifty per cent effective concentration, or compound concentration required to inhibit retrovirus-induced cytopathicity in MT-4 cells by 50%.

† Fifty per cent cytotoxic concentration, or compound concentration required to reduce MT-4 cell viability by 50%.

Table 2. Inhibitory effects of metalloporphyrin–ellipticine complexes on syncytium formation between persistently HIV-1- or HIV-2-infected HUT-78 cells and uninfected MOLT-4 cells

	EC_{50}* (μg/mL)	
Compound	HUT-78/HIV-1 + MOLT-4	HUT-78/HIV-2 + MOLT-4
1-Mn	20	>20
2-Mn	7	>20
3-Mn	20	—
3-Zn	70	>100
4-Mn	10	—
5-Mn	40	>40

* Fifty per cent effective concentration, or compound concentration required to inhibit the formation of syncytia between HIV-infected HUT-78 cells and MOLT/4 cells by 50%.

The compounds were examined for their inhibitory effect on syncytium formation between persistently HIV-1- or HIV-2-infected cells and uninfected MOLT-4 (clone 8) cells. Syncytium formation between HIV-1-infected HUT-78 cells and MOLT-4 cells was inhibited by the compounds at concentrations ranging from 7 to 70 μg/mL, whereas syncytium formation between HIV-2-infected HUT-78 cells and MOLT-4 cells was not inhibited by any of the compounds at subtoxic concentrations (Table 2).

Using a flow cytometric method [23], we have demonstrated previously that both polyanionic [28–31] and polycationic [32] compounds are able to block HIV binding to $CD4^+$ cells. This finding apparently extends to the metalloporphyrin–ellipticin complexes. At a concentration of 25 μg/mL, **1-Mn** and **2-Mn** were found to inhibit HIV-1 binding to MT-4 cells by 60%.

The best compounds were also evaluated for their ability to inhibit the binding of mAbs (OKT44/Leu3a) to the CD4 receptor of MT-4 cells. None of the compounds (at a concentration of 50 μg/mL) interfered with the binding of OKT4A/Leu3a mAb to CD4.

We then examined whether the metalloporphyrin–ellipticine complexes may interact with the viral gp120 glycoprotein that is involved in HIV binding to the cells and syncytium formation. to this end, we used persistently HIV-1-infected HUT-78 cells and mAbs recognising the V3 region of gp120 which is assumed to play a critical role in syncytium formation [33]. None of the compounds had an inhibitory effect on the binding of anti-gp120, even when used at a concentration of up to 50–100 μg/mL.

The test compounds **1-Mn** and **2-Mn** were also evaluated for their inhibitory effect on HIV-1 RT activity, using poly(A).oligo(dT) as the template/primer and [^{3}H-*methyl*]-dTTP as the substrate. Both compounds were found to inhibit RT activity, their 50% inhibitory concentration being 10 and 11 μg/mL, respectively.

Discussion

Activity of the metalloporphyrin–ellipticine complexes was observed against HIV-1(III_B) and SIV(MAC_{251}), but not HIV-2(ROD). Also, **1-Mn**, **2-Mn** and **3-Zn** were found to be inactive against Moloney murine sarcoma virus-induced transformation of murine C3H/3T3 cells at subtoxic concentrations (data not shown). Of all the test compounds, **3-Mn** showed the highest selectivity. Its selectivity index, as based on the CC_{50}/EC_{50} ratio, was 30 (Table 1).

The compounds were also found to be inhibitory to syncytium formation between persistently HIV-1-infected HUT-78 cells and uninfected MOLT-4 cells. Furthermore,

they proved inhibitory to the HIV-1-associated RT and to the binding of HIV-1 to the cells.

It is unclear, however, how the test compounds achieved their inhibitory effect on virus–cell binding. They did not interfere with either binding of OKT4A/Leu3a mAb to the CD4 receptor of MT-4 cells or binding of specific mAb to the V3 region of gp120. This contrasts with the sulfated polysaccharides [26], sulfated polymers [28], sulfated cyclodextrins [29], polycarboxylates [30] and polyhydroxycarboxylates [31], which all interfere with the V3 region of gp120.

Although the compounds are inhibitory to HIV-1 RT, it is not certain that this inhibitory effect contributes to their antiviral activity, since the latter may be mediated solely by interference with the viral adsorption step. It would now seem imperative to introduce further modifications into the chemical structure of the compounds so as to improve their anti-HIV selectivity and to pinpoint their molecular target of antiviral action.

Acknowledgements—L. Ding is indebted to the CNRS for a Research Fellowship. Pierre Fabre Médicaments (Castres), the Région Midi-Pyrénées and the French Agency for AIDS Research (ANRS) are acknowledged for financial support. This work has also been supported by the AIDS Basic Research Programme of the European Community and by grants from the Belgian Fonds voor Geneeskundig Wetenschappelijk Onderzoek (Project 3.0097.87), the Janssen Research Foundation and the Belgian Geconcerteerde Onderzoeksacties (Project 90/94-2). Dr G. Etemad-Moghadam provided a sample of compound **5**. We thank Ann Absillis and Lizette van Berckelaer for excellent technical assistance, and Christian Callebaut for fine editorial help.

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