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Anti-HIV activities of anionic metalloporphyrins and related compounds

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Summary

Various water-soluble polysulphonated and polycarboxylated porphyrins and some of their metallated derivatives have been prepared and their antiviral properties against human immunodeficiency virus (HIV-1, HIV-2), simian immunodeficiency virus and other viruses are reported. Besides these polyanionic compounds, two new series of porphyrins were included and studied from the perspective of bio-availability modulation: (i) acetylsulphonamido derivatives endowed with weak acidity properties (deprotonation gives the corresponding anionic derivatives in a pH range 4.5–8.5) and (ii) compounds with the anionic charge transiently masked by esterification (acetoxymethyl- and pivaloyloxymethylesters). Among the more active compounds in inhibiting HIV-induced cytopathic effects, the sulphonated and carboxylated porphyrin complexes were found to interact directly with the HIV protein gp120 and not with the CD4 cellular receptor.

Keywords: Anionic metalloporphyrin; prodrug; antiviral; anti-HIV; gp120 interaction.

Introduction

Among the drugs licensed in the US for clinical use in the treatment of patients with AIDS are five dideoxynucleoside analogues: zidovudine [3′-azido-2′,3′-dideoxythymidine or azidothymidine (AZT); Langtry & Campoli-Richards, 1989; Volberding *et al.*, 1990], didanosine [2′,3′-dideoxyinosine (ddI); Faulds & Brogden, 1992], zalcitabine [2′,3′-dideoxycytidine (ddC); Whittington & Brogden, 1992], stavudine [2′,3′-didehydro-2′,3′-dideoxythymidine (D4T); Riddler *et al.*, 1995] and lamivudine [2′,3′-dideoxy-3′-thiacytidine (3TC); Schinazi *et al.*, 1992]. Most of these drugs exhibit some severe toxic side-effects, and the rapid emergence of resistant human immunodeficiency virus type 1 (HIV-1) variants should be considered a limitation to their clinical usefulness (St Clair *et al.*, 1991; De Clercq, 1994, 1995a). Consequently, new molecules are needed for the treatment of AIDS patients and asymptomatic seropositive HIV carriers (for recent review articles on antiretroviral agents, see Sarin, 1988; Mitsuya *et al.*, 1990; De Clercq, 1991, 1995a,b; Johnston & Hoth, 1993; Yarchoan *et al.*, 1993).

Two phases of the HIV replication cycle can be distinguished: (i) an early phase, from the attachment of virions to the target cells to the formation of proviral DNA and (ii) a late phase with the expression of viral proteins and the release of new viral particles. Accordingly, two categories of antiviral agents can be discerned. Reverse transcriptase inhibitors (nucleoside analogues and benzodiazepine derivatives) belong to the first category, whereas antisense oligonucleotides, HIV protease inhibitors and glycosylation inhibitors correspond to the second category (Mitsuya *et al.*, 1990; De Clercq, 1991). However, prevention of the binding of HIV to target cells, namely the interaction of the viral envelope protein gp120 with the T cell receptor protein CD4 (Reed & Kinzel, 1991), is also a possible therapeutic approach and has been demonstrated for the soluble form of CD4 (Fisher *et al.*, 1988). The plasma half-life of soluble CD4 is longer when attached to human immunoglobulin (Capon *et al.*, 1989).

Polyanionic molecules such as sulphated polysaccharides (e.g. dextran sulphate), polysulphonates (e.g. suramin) and polycarboxylates (e.g. aurintricarboxylic acid) (De Clercq, 1995a) have been shown to block the

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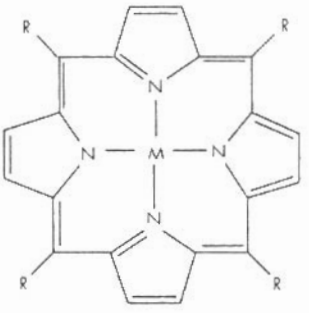
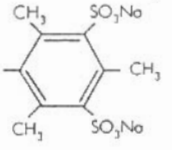
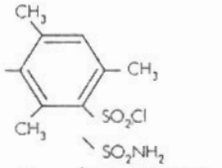
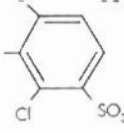
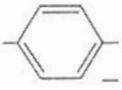
binding of HIV virions to CD4⁺ cells. Aurintricarboxylic acid predominantly interacts with the CD4 receptor (Schols *et al.*, 1989), whereas dextran sulphate has a shielding effect on the viral gp120 protein (Schols *et al.*, 1990). Despite the low clinical activity of dextran sulphate when orally administered (Abrams *et al.*, 1989), this polyanionic molecule is the prototype for other anionic derivatives that can block viral replication by inhibiting viral binding. Inhibition of viral capsid assembly might be an alternative mechanism, as was shown recently for a sulphonic acid derivative (Teschke *et al.*, 1993).

Polyanionic coordination complexes with a redox active metal centre might be considered as possible candidates for a new category of viral binding inhibitors, combining the effect of negative charges at the periphery of the ligand with a redox active centre in the middle of the complex, in order to create irreversible damage to binding proteins of viral particles during the entry process. The availability or the relatively easy access to a number of synthetic porphyrins, the known low toxicity for some of their anionic derivatives (Megnin *et al.*, 1987; Meng *et al.*, 1994) and earlier reports on the screening of porphyrins as antiviral agents against HIV (Ding *et al.*, 1992; Dixon *et al.*, 1992; Neurath *et al.*, 1992; Debnath *et al.*, 1994) prompted us to study anti-HIV-1 and anti-HIV-2 activities of a large series of anionic porphyrin ligands and their corresponding metallated derivatives. Sulphonated manganese- and iron-porphyrins have been developed in our laboratory for mimicking ligninase (Labat & Meunier, 1989) or chloroperoxidase (Labat & Meunier, 1990), and as biomimetic catalysts for the oxidation of pollutants (Labat *et al.*, 1990) or drugs (Bernadou *et al.*, 1991; Vidal *et al.*, 1993; Gaggero *et al.*, 1994). The manganese complex of *meso*-tetrasulphonatophenylporphyrin (**1-Mn**; Table 1) has also been studied by several groups as a contrast-enhancing agent for tumour imaging by nuclear magnetic resonance (NMR) imaging (Megnin *et al.*, 1987; Fiel *et al.*, 1990). No toxicity of **1-Mn** was observed to the human breast cancer cell line MCF-7 at 0.1 mg mL^{-1} (Megnin *et al.*, 1987).

We describe in the present article the synthesis of various water-soluble polyanionic porphyrins and some of their metallated derivatives, and report their antiviral properties against HIV-1, HIV-2, simian immunodeficiency virus (SIV) and other viruses. Besides these polysulphonated and polycarboxylated compounds, two new series were included in order to modulate bioavailability. A series of acetylsulphonamido derivatives endowed with weak acidity properties (deprotonation gives anionic derivatives in a pH range 4.5–8.5) was expected to present improved bioavailability, especially in oral administration. Since anionic metalloporphyrins are negatively charged molecules which are generally poorly absorbed, we also evaluated a second series of compounds with the negative charge transiently masked by esterification. Because simple alkyl or aryl esters are slowly hydrolysed, we also prepared acyloxymethylesters (acetoxymethyl- and pivaloyloxymethylesters) which are known to be rapidly hydrolysed after absorption (Daehne *et al.*, 1970; Shimizu *et al.*, 1993).

Table 1. Structure of the porphyrin ligands and their metallated derivatives.



R	M =	H_2	Mn	Fe	Ni
 SO_3Na		1	1- Mn	1- Fe	–
SO_2Cl		[2]	–	–	–
SO_2NH_2		3	3- Mn	4- Fe	3- Ni
$SO_2NHCOCH_3$		4	4- Mn	4- Fe	4- Ni
		5	5- Mn	5- Fe	–
(bromo-TMPS)[a]		[6]	6- Mn	6- Fe	–
		[7]	–	–	–
		8	8- Mn	8- Fe	8- Ni
		9	9- Mn	9- Fe	9- Ni
		[10]	10- Mn	10- Fe	–
 $COOH$		11	[11- Mn]	11- Fe	11- Ni
$COOCH_2OCOtBu$		12	12- Mn	12- Fe	12- Ni
$COOCH_2OCOCH_3$		13	13- Mn	13- Fe	13- Ni

[a]Same structure as **5** but the pyrroles are brominated on all the β-positions [TMPS, *meso*-tetrakis(3,5-disulphonatomesityl)-porphyrin]. Compounds between square brackets are synthesis intermediates and were not tested for antiviral activity.

Material and Experimental Procedures: Chemistry

Except general remarks on the procedures of porphyrin synthesis, all details on instrumentation and most of the protocols of synthesis are available from the Editors as supplementary data (requests should quote the full journal citation). Only the preparation of **2**, **3**, **4** and **4-Mn** (as examples of sulphonamide derivatives) and **13** and **13-Mn** (as examples of labile esters) are presented here in detail. Molecular weights of the tested compounds shown in Tables 3–5 (including axial ligands for metallated derivatives and solvent of crystallization if present) were as follows:

1-Mn, 1382; **1-Fe**, 1383; **3**, 1025; **3-Mn**, 1188; **3-Fe**, 1092; **3-Ni**, 1097; **4**, 1135; **4-Mn**, 1320; **4-Fe**, 1303; **4-Ni**, 1210; **5**, 1779; **5-Mn**, 1832; **5-Fe**, 1833; **6-Mn**, 2301; **6-Fe**, 2302; **8**, 1135; **8-Mn**, 1355; **8-Fe**, 1202; **8-Ni**, 1192; **9**, 1340; **9-Mn**, 1488; **9-Fe**, 1452; **9-Ni**, 1396; **10-Mn**, 1466; **10-Fe**, 1467; **11**, 790; **11-Fe**, 880; **11-Ni**, 846; **13-Mn**, 1209.

Preparation of unmetallated porphyrins

Parent hydrophobic porphyrins. *Meso*-tetramesitylporphyrin (H_2TMP) and *meso*-tetrakis(2,6-dichlorophenyl)porphyrin (H_2TDCPP) were prepared from pyrrole and the corresponding aldehyde by the porphyrinogen method (Lindsey & Wagner, 1989; Hoffmann *et al.*, 1992). *Meso*-tetramesityl-β-octabromo-porphyrin (H_2Br_8TMP) was obtained by bromination of tetramesitylporphyrin (Hoffmann *et al.*, 1992).

Anionic hydrophilic porphyrins. **1** was supplied by Strem Chemicals and **11** by Aldrich. Other sulphonated porphyrin ligands **5**, **6** and **10** were obtained by sulphonation of the corresponding ligand.

Sulphonamide derivatives (examples of 2, 3 and 4)

(i) Meso-*tetrakis(4-chlorosulphonylphenyl)porphyrin 2.* H_2TPP (200 mg, 0.33 mmol) was dissolved in 20 mL of CH_2Cl_2. Then 2.4 mL of chlorosulphonic acid were added dropwise with stirring at 0°C. When the initial formation of hydrogen chloride stopped, the reaction mixture was warmed to room temperature, stirred for 0.5 h, and then heated to 55°C for 1 h. The reaction mixture was poured slowly into a 100 mL beaker filled with crushed ice. The CH_2Cl_2 layer was separated and washed with cold water until the pH of the washing water reached 5–6 (about three consecutive washings). The organic phase was dried over anhydrous sodium sulphate and evaporated to dryness. The crude product was then kept under vacuum for the following reaction.

(ii) Meso-*tetrakis(4-sulphonamidophenyl)porphyrin 3.* The crude product from the chlorosulphonation (about 300 mg of **2**) was refluxed for 1 h with a large excess (14 mL) of 28% aqueous ammonia. After cooling to room temperature and adding cold water, the violet solid was filtered with suction and washed several times with water. The residue was then purified on a silicagel column (the eluent was acetone/hexane from 75/25 to 99/1) and dried under vacuum (40% yield). Anal. data: UV-visible (in DMF) λ nm (ε, mM^{-1} cm^{-1}): 420 (395), 514 (15.4), 548 (2.7) and 592 (5.2). ^{1}H NMR (250 MHz, DMSO-d_6): δ 8.97 (s, 8 H, H-pyrrole), 8.57 (d, 8 H, *J* 8.2 Hz, m-H-Ar), 8.41 (d, 8 H, *J* 8.2 Hz, o-H-Ar), 7.87 (s, 8 H, NH_2), −2.14 (s, 2 H, NH-pyrrole). MS data (FAB$^+$/MNBA), m/z 931 (MH^+).

(iii) Meso-*tetrakis(4-acetylsulphonamidophenyl)porphyrin 4.* To a mixture of 300 mg (0.322 mmol) of **3** in 10 mL of pyridine and 7.5 mL of glacial acetic acid, was added 7.5 mL (0.105 mol) of acetyl chloride. The resulting solution was refluxed at 55°C for 4 h. After cooling to room temperature and adding cold water, the porphyrin was filtered with suction and washed with water. The precipitate was dissolved in 5% aqueous sodium bicarbonate solution. The product was precipitated by acidification with acetic acid up to pH 4, then filtered with suction and washed twice with water. The residue was dissolved in DMF and precipitated by diethylether. The final product was obtained as violet powder and dried under vacuum (76% yield). Anal. data: UV-visible (in DMF) λ nm (ε, mM^{-1} cm^{-1}): 420 (520), 514 (23), 548 (13) and 592 (7.4). ^{1}H NMR (250 MHz, DMSO-d_6): δ 12.54 (s, 4 H, NHCO), 8.97 (s, 8 H, H-pyrrole), 8.62 (d, 8 H, *J* 8.3 Hz, m-H-Ar), 8.49 (d, 8 H, *J* 8.3 Hz, o-H-Ar), 2.25 (s, 12 H, CH_3), −2.85 (s, 2 H, NH-pyrrole). MS data (FAB$^+$/MNBA), m/z 1099 (MH^+).

Tetracarboxyphenylporphyrin labile esters (example of 13)

Meso-*tetrakis(4-acetoxymethylcarboxylatophenyl)porphyrin 13.* To a solution of 400 mg (0.51 mmol) of **11** in 12 mL of DMF and 1.19 g (4.05 mmol) of N,N′-dicyclohexyl-4-morpholine carboxamidine, 394 µL (4.05 mmol) of bromomethyl acetate were added. After 1 h of stirring at room temperature, 5 vol. of CH_2Cl_2 were added. The solution was washed successively with water, 5% aqueous sodium bicarbonate solution and water, then dried over anhydrous sodium sulphate and evaporated. The residue was purified on a dry silicagel column (methanol/CH_2Cl_2 from 50/50 to 70/30 (v/v) as eluent). The final product was obtained by precipitation from a CH_2Cl_2 solution by addition of methanol and dried under vacuum (65% yield). Anal. data: UV-visible (in CH_2Cl_2) λ nm (ε, mM^{-1} cm^{-1}): 420 (440), 514 (30.6), 550 (19.5) and 588 (16.6). ^{1}H NMR (250

MHz, $CDCl_3$): δ 8.80 (s, 8 H, H-pyrrole), 8.46 (d, 8 H, *J* 8.2 Hz, m-H-Ar), 8.32 (d, 8 H, *J* 8.2 Hz, o-H-Ar), 6.17 (s, 8 H, OCH_2O), 2.24 (s, 12 H, CH_3), −2.85 (s, 2 H, NH-pyrrole). MS data (FAB⁺/MNBA), m/z 1079 (MH^+).

Metallated porphyrins

Metallations were monitored by UV-visible spectroscopy. The exact nature of the axial ligands of the different metalloporphyrins was highly dependent on the counter-ions or neutral ligands present in the reaction mixture or during the final work-up. In addition it must be noted that these axial ligands are labile and might easily be replaced by other potential ligands in cell culture medium. Also, the nature of these ligands depends on pH values as discussed by Zipplies *et al.* (1986) and Vidal *et al.* (1993): a water molecule is present in axial position at low pH (below 7) and an hydroxo ligand at pH>7.

Metallation with manganese of porphyrins 4 and 13

(i) Meso-*tetrakis(4-acetylsulphonamidophenyl)porphyrinato-manganese(III) 4-Mn*. To a solution of porphyrin 4 (0.054 mmol) in 5 mL DMF was added 36 µL of 2,4,6-collidine (0.54 mmol) and an excess of $Mn(OAc)_2.4H_2O$ (0.54 mmol). The mixture was heated at 140°C for 2 h. The completion of the reaction was followed spectrophotometrically. The reaction mixture was then cooled to room temperature and the solvent evaporated to dryness. The crude metallated product was dissolved in 5% aqueous sodium bicarbonate solution, precipitated by acidification with acetic acid at pH 4, washed several times with water and, finally, reprecipitated in DMF with diethylether and dried under vacuum (65% yield). Anal. data: UV-visible (in DMF) λ nm (ε, mM^{-1} cm^{-1}): 380 (55.3), 402 (58.7), 422 (48.0), 468 (141), 568 (14.5), 602 (11.9). MS data (FAB⁺/MNBA), m/z 1151 (M^+).

(ii) Meso-*tetrakis(4-acetoxymethylcarboxylatophenyl)porphyrinato-manganese(III) 13-Mn*. The procedure was the same as described above, except that the crude product was purified on a neutral alumina column (methanol/CH_2Cl_2 from 50/50 to 70/30 (v/v) as eluent) and the final metallated product was obtained by precipitation with hexane in CH_2Cl_2 (50% yield). Anal. data: UV-visible (in CH_2Cl_2) λ nm (ε, mM^{-1} cm^{-1}): 376 (47.4), 400 (42.3), 474 (98), 574 (6.1), 612 (6.2). MS data (FAB⁺/MNBA), m/z 1131 (M^+).

Apparent partition coefficients and determination of pK_a for 4, 4-Mn, 4-Fe, 4-Ni, 9, 9-Mn, 9-Fe and 9-Ni

The apparent partition coefficients at pH 7.4 were determined by introducing 3–10 µL of each porphyrin solution (1 mM in DMF) into a n-octanol/phosphate buffer (0.66 mM, pH 7.4) mixture. The two phases (1.5 mL each) were previously mutually saturated. The combined phases were then shaken by vortexing for 1 min, centrifuged for 20 min at 4000 r.p.m. to separate the layers, and each phase was analysed by spectrophotometry. The absorbance at the Soret band was determined for both aqueous (A_w) and octanolic (A_{oct}) fractions. Apparent partition coefficient (P) values were then calculated as A_{oct}/A_w and are listed in Table 2.

Table 2. Apparent partition coefficients (P) at pH 7.4 and pK_a values of compounds **4**, **9** and their metallated derivatives.

Compound	P	pK_a
4-Mn	<0.01	4.6
4	<0.01	6.3
4-Fe	0.19	6.5
4-Ni	0.04	6.7
9-Mn	1.3	7.5
9	3.5	7.7
9-Fe	5.7	8.1
9-Ni	22	8.3

The pK_a values were determined by introducing 3–10 µL of each porphyrin solution (1 mM in DMF) into the octanol/phosphate buffer (0.66 mM at the chosen pH) system, the two solvents being previously mutually saturated. The resulting mixture was then shaken by vortexing for 1 min, centrifuged for 20 min at 4000 r.p.m. and each phase was then analysed by spectrophotometry. The pH at which the same quantity of porphyrin derivative was observed in each phase (50/50) was considered as the pK_a value.

Hydrolysis of compounds 12 and 13 in various media

Stability against hydrolysis of esters **12** and **13** has been tested in various media: 2 mL of 2 µM solution of **12** or **13** in CH_2Cl_2 were added to 1 mL of an aqueous phase containing the chosen buffer or the esterase and maintained under magnetic stirring at 37°C for 30 min or 6 h. After decantation of the mixture, the percentage absorbance decay of the organic phase was considered as the percentage hydrolysis. Experiments were conducted in triplicate. The percentage hydrolysis in 0.1 M phosphate buffer pH 7.2, 0.1 M Tris buffer pH 9.0, 1 M KOH or using carboxyl esterase (80 U mL^{-1}; from porcine liver; Sigma) in phosphate buffer pH 7.4 were for 30 min (or 6 h) incubation: for **12**, 0 (0), 0 (0), 50 (100) and 29 (62), respectively; for **13**, 0 (3), 25 (51), 98 (100) and 56 (99), respectively.

Materials and Experimental Procedures: Virology

Antiretroviral activity and cytotoxicity

Antiretroviral activity against HIV-1_{IIIB} (Popovic *et al.*, 1984), HIV-2_{ROD} (Barré-Sinoussi *et al.*, 1983) or SIV_{MAC251} (Daniel *et al.*, 1987) and cytotoxicity of test compounds in human MT-4 lymphocyte cells (Table 3) were measured. The methodology of the anti-HIV assays has been described previously by Pauwels *et al.* (1988). Briefly, MT-4 cells were suspended in fresh culture medium and infected with HIV-1, HIV-2 or SIV at 100

Table 3. Antiretroviral and cytotoxic activity of test compounds in human MT-4 lymphocyte cells.

	EC_{50}[a]			CC_{50}[b]	SI[c]		
Compound	HIV-1_{IIIB}	HIV-2_{ROD}	SIV_{MAC251}	MT-4	HIV-1_{IIIB}	HIV-2_{ROD}	SIV_{MAC251}
1-Mn	46	29	120	>200	>4.3	>6.9	>1.7
1-Fe	2	8	18	>40	>20	>5.0	>2.2
3	18	5	-	70	3.9	14	-
3-Mn	>200	>80	-	>200	×1	×2.5	-
3-Fe	125	150	-	>250	>2.0	>1.7	-
3-Ni	60	19	-	>250	>4.2	>13.2	-
4	>35	>50	-	50	<1.4	<1	-
4-Mn	17	160	-	>215	>12.6	>1.3	-
4-Fe	2	10	>63	>100	>50	>10	×1.6
4-Ni	18	55	-	>250	>13.9	>4.5	-
5	1	1	≥4	4	4	4	<1
5-Mn	36	33	8	>100	>2.8	>3.0	>12.5
5-Fe	4	4	1	>100	>25	>25	>100
6-Mn	10	100	15	>100	>10	>1	>6.7
6-Fe	16	9.5	10	>200	>12.5	>21.1	>20
8	>80	>80	-	80	<1	<1	-
8-Mn	20	>250	-	>250	>12.5	×1	-
8-Fe	>250	>250	-	>250	×1	×1	-
8-Ni	75	70	-	>250	>3.3	>3.6	-
9	9	75	-	75	8.3	<1	-
9-Mn	18	≥200	-	≥240	≥13.3	×1.2	-
9-Fe	12	>190	-	>220	>18.3	×1.2	-
9-Ni	4	>200	-	>230	57.5	×1.2	-
10-Mn	14	20	40	≥100	≥7.1	≥5	≥2
10-Fe	5	10	8	40	8	4	5
11	0.9	>23	>8	23	25.6	<1	<2.9
11-Fe	0.5	4	12	>90	>180	>22.5	>7.5
11-Ni	1	8.5	8	>90	>90	>10.6	>11.3

[a] 50% effective concentration (μg ml^{-1}); concentration required to inhibit retrovirus-induced cytopathicity in MT-4 cells by 50%.
[b] 50% cytotoxic concentration (μg ml^{-1}); concentration required to reduce MT-4 cell viability by 50%.
[c] Selectivity index; ratio of CC_{50} to EC_{50}

Table 4. Antiviral assays with **5-Fe**, **11**, **11-Fe**, **11-Ni** and reference drugs.

The reference drugs used were bromovinyldeoxyuridine (BVDU); (S)-9-(2,3-dihydroxypropyl)adenine [(S)-DHPA]; ribavirin; carbocyclic 3-deazaadenosine (C-c3Ado); acyclovir (ACV); dextran sulphate (DS5000); and ganciclovir (GCV).

	5-Fe	11	11-Fe	11-Ni	BVDU	DS5000	(S)-DHPA	Ribavirin	C-c3Ado
Vero cells									
Minimum cytotoxic concentration[a] ($\mu g\ mL^{-1}$)									
	≥200				>400		>400	>400	>400
Minimum inhibitory concentration[b] ($\mu g\ mL^{-1}$)									
Parainfluenza-3 virus	>150				>400		350	70	2
Reovirus-1	>100				>400		220	180	1.5
Sindbis virus	5				>400		≥230	70	35
Coxsackie virus B4	>150				>400		>400	>400	≥350
Semliki forest virus	40				>400		>400	230	>400
E_6SM cells									
Minimum cytotoxic concentration[a] ($\mu g\ mL^{-1}$)									
	≥300	>400	>400	>400	≥300	>400	≥400	≥400	≥400
Minimum inhibitory concentration[b] ($\mu g\ mL^{-1}$)									
VV	85	>400	>400	>400	1.5	>400	55	40	2
VSV	>85	>400	150	>400	>350	10	40	200	0.6
HSV-1 strains									
TK⁻B2006	50	70	70	70	0.5	2	≥250	>300	≥200
TK⁻/TK⁺VMW1837	20	70	70	70	0.5	2	110	60	30
KOS	20	70	70	70	0.015	2	>200	≥160	≥140
HSV-2 (G)	20	70	70	70	>100	4	>300	>250	>300
HeLa cells									
Minimum cytotoxic concentration[a] ($\mu g\ mL^{-1}$)									
	≥150	≥400	>400	≥400	≥400	>400	>400	≥150	>400
Minimum inhibitory concentration[b] ($\mu g\ mL^{-1}$)									
VSV	70	300	150	150	>300	7	180	30	2
Coxsackie virus B4	>100			>400	>400		>400	55	>400
Poliovirus-1	>100				>400		>400	110	>400

	5-Fe	11	11-Fe	11-Ni	BVDU	DS5000	ACV	GCV
HEL cells								
Cytotoxicity (ID_{50}[c] [$\mu g\ mL^{-1}$])								
	150	>50	>50	>50	190	>50	>200	>50
Antiviral activity (ID_{50}[d] [$\mu g\ mL^{-1}$])								
VZV strains								
TK⁺ OKA	12				0.004		0.39	0.28
TK⁺ YS	10				0.01		0.65	0.43
TK⁻ 07/1	4.5				37		16	1.5
TK⁻ YS/R	5.5				56		7.6	1
CMV strains								
AD169	25	1.4	2	2	200	0.85	27	1.6
Davis	6	1	2	1.6	200	0.9	26	1.7

[a] Required to cause a microscopically detectable alteration of normal cell morphology.

[b] Required to reduce virus-induced cytopathogenicity by 50%.

[c] 50% Inhibitory concentration; concentration required to reduce virus plaque formation by 50%. Virus input was 20 p.f.u.

[d] 50% Inhibitory concentration; concentration required to reduce cell growth by 50%.

$CCID_{50}$ (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 and mixed with 100 µL of the appropriate dilutions of the test compounds. After 5 days, the number of viable cells was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method. 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.

Other antiviral assays

Antiviral assays were also carried out with **5**, **5-Mn**, **5-Fe**, **11**, **11-Fe**, **11-Ni** and reference drugs (Table 4). These assays were based on inhibition of virus-induced cytopathicity in either E_6SM [herpes simplex virus type 1 (HSV-1), HSV-2, vaccinia virus (VV), vesicular stomatitis virus (VSV)], human embryonic lung (HEL) [varicella-zoster virus (VZV), cytomegalovirus (CMVX)], HeLa (Coxsackie virus B4, poliovirus-1) or Vero (parainfluenza 3 virus, reovirus-1, Sindbis virus, Coxsackie virus B4, Semliki forest virus) cell cultures, following previously established procedures (De Clercq *et al.*, 1980, 1986, 1987; Baba *et al.*, 1987). Briefly, confluent cell cultures in microtitre trays were inoculated with 100 $CCID_{50}$ of virus. After a 1 h virus adsorption period, residual virus was removed and the cell cultures were incubated in the presence of varying concentrations (400, 200, 100, etc. µg mL^{-1}) of the test compounds. Viral cytopathic effects were recorded as soon as they reached completion in the virus-infected cell cultures that were not treated with the test compounds.

Inhibition of syncytium formation

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 microtitre trays. To each well were then added 5×10^4 MOLT-4 (clone 8) cells (in 50 µL) and an appropriate concentration of the test compound (in 100 µL). The mixed cells were cultured at 37°C in a CO_2-controlled atmosphere. After 16–20 h of cocultivation the number of syncytia was examined microscopically (Table 5).

Glycoprotein gp120 immunofluorescence assay

This method has been described elsewhere in detail (Schols *et al.*, 1990). Briefly, HUT-78/HIV-1 cells (2×10^5) in 100 µL of culture medium were incubated with the compounds at various concentrations for 15–20 min, washed twice to remove residual compound, stained with anti-gp120 MAb (9284, Du Pont) at 37°C, washed twice, incubated with fluorescein isothiocyanate (FITC)-conjugated $F(ab')_2$ fragments of rabbit anti-mouse IgG [RaM-IgG-$F(ab')_2$FITC] (Prosan, Ghent, Belgium) at 37°C, washed twice, resuspended in 0.5 mL of buffer, and analysed by flow cytometry (Fig. 1).

Table 5. Inhibitory effect of test compounds against giant cell formation between HUT-78/HIV-1 or HUT-78/HIV-2 cells and uninfected MOLT-4 cells.

	EC_{50}[a] (µg mL^{-1})	
	HUT-78/HIV-1	HUT-78/HIV-2
1-Mn	>100	>100
1-Fe	7	40
4-Fe	9	56
6-Mn	70	100
6-Fe	20	10
10-Fe	>20	>20
11	10	59
11-Fe	37	60
11-Ni	37	60
13-Mn	>125	>125
Dextran sulphate[b]	15	0.03

[a] 50% effective concentration; concentration required to inhibit syncytium formation by 50%.
[b] Molecular weight 5000.

CD4 immunofluorescence assay

This method has been described elsewhere in detail (Schols *et al.*, 1989). MT-4 cells were briefly incubated in the presence or absence of test compound. The cells were then stained with MAb anti-Leu3a-PE or Simultest immune monitoring kit control (FITC-labelled IgG_1 and phycoerythrin-labelled IgG_2) (Becton Dickinson) for the negative control, washed once, fixed in 1% paraformaldehyde, and examined cytofluorometrically.

Results

Chemistry

Porphyrin **1** is a commercial product. Table 1 shows the structures of the porphyrin derivatives. The hydrophobic derivatives *meso*-tetramesitylporphyrin (H_2TMP), *meso*-tetrakis(2,6-dichlorophenyl)porphyrin (H_2TDCPP), *meso*-tetramesityl-β-octabromoporphyrin (H_2Br_8TMP) and the sulphonated compound **6** were prepared as previously described by Hoffmann *et al.* (1990, 1992). The

methods for synthesis of porphyrins **5** (Hoffmann *et al.*, 1990; Pattou *et al.*, 1994), **10** (Turk & Ford, 1991; Campestrini & Meunier, 1992) and their metallated complexes were improved. For instance, reaction time and temperature had to be strictly controlled in order to avoid over-sulphonation of the ligand. Owing to the heterogeneity of the reaction mixture, highly efficient magnetic stirring was also a key point in improving the sulphonation. Moreover, in the special case of **10**, treatment of H_2TDCPP with oleum 18–23% under the conditions used for H_2TMP led to an hydrosoluble porphyrin bearing four sulphonato groups per molecule, but, as was shown by ^{1}H NMR spectroscopy, there was no selectivity of sulphonation on the *meta* positions of the phenyl groups compared to the *para* positions. On the contrary, the sulphonation of H_2TDCPP in relatively mild conditions (anhydrous H_2SO_4) yielded in a reproducible way the expected product **10** which was sulphonated only at the *meta* positions of each dichlorophenyl group. After the sulphonation step, the main difficulty was to recover the hydrosoluble sulphonated porphyrin from a huge amount of sodium sulphate. Combination of extraction with methanol and centrifugation allowed isolation of these ligands with a minimum contamination of salt. In the following step, metallation of the sulphonated porphyrins with manganese or iron was performed in a very simple way in water with a rather low excess of metal salt equivalents (2–5). Purification by chromatography over a chelating resin yielded the metalloporphyrins without contamination by free metal salts.

Additionally, two series of original compounds derived from sulphonic or carboxylic porphyrins were prepared:

(i) Porphyrins substituted in the *meso* phenyl or mesityl groups by a chlorosulphonic function were successively converted to sulphonamide and acetylsulphonamide derivatives (and their metallated derivatives). The sulphonyl chloride derivatives **2** and **7** were directly obtained in good yields by reacting H_2TPP or H_2TMP with chlorosulphonic acid. It must be noted that, under our conditions, the chlorosulphonation of H_2TMP gave only **7**, not the dichlorosulphonyl derivative. Alternatively, neither this latter one nor compound **2** could be obtained in satisfactory yields by direct treatment of **1** or **5** with thionyl chloride. Reaction of **2** or **7** with ammonia gave the sulphonamide compounds **3** or **8**, which, in a further step, were transformed into the acetylsulphonamide derivatives **4** and **9** by reacting with acetyl chloride. Pure compounds **8** and **9** were in fact a mixture of atropo-isomers, as was demonstrated by careful NMR analysis (data not shown; the isomers existed in a statistical distribution). The lipophilic character of **4** and **9** (and their metallated derivatives) in slightly acidic medium gives hope of an improved absorption in such pH conditions *in vivo* (e.g. in the stomach). Depending on the ligand structure and on the complexed metal, the pK_a values ranged from 4.6 to 8.3 (Table 2).

(ii) Labile esters of **11** (and their metallated derivatives) were prepared. They are prodrug types which have acetoxymethyl (**13** and **13-M**) or pivaloyloxymethyl (**12** and **12-M**) ester groups and are expected to be easily hydrolysed *in vivo* to release the active anionic metalloporphyrins **11** or **11-M**, as well as formaldehyde and acetic or pivalic acid as indicated:

Por-CO-O-CH_2-O-CO-tBu•H_2O→Por COOH•HCHO•tBu-COOH

Short kinetic experiments, intended to test the stability of these esters, were carried out at 37°C over a time period of 0.5–6 h in conditions described in the Methods. The pivaloyloxymethyl esters appeared much more resistant to enzymatic and principally chemical hydrolysis than the corresponding acetyloxymethyl ones: as an example, in the presence of carboxyl esterase at pH 7.4 and after 6 h incubation, hydrolysis of **13** was almost complete whereas it was only 62% for **12**. At pH 9, no hydrolysis could be detected after 6 h for **12** when **13** was hydrolysed up to 50%.

Virology

Antiretroviral activity. HIV-1, HIV-2 and SIV inhibition and toxicity in MT-4 cells for 36 tetrasubstituted phenylporphyrins were studied. The main results are given in Table 2. The six most active compounds were tested for their activity against a range of other viruses and compared to reference drugs (main results in Table 3).

We must note firstly that the free ligands appeared systematically more cytotoxic than the corresponding metallated derivatives (Table 3). For example, **5** (CC_{50} 4 μg mL^{-1}) was much more cytotoxic than **5-Mn** or **5-Fe** (>100); this was also true, but the difference was not as marked for **3**, **4**, **8**, **9** and **11** compared to **3-M**, **4-M**, **8-M**, **9-M** and **11-M**, respectively (M indicates Mn, Fe or Ni). All of these compounds were simultaneously evaluated for their inhibitory effect on the cytopathic effect of HIV-1, HIV-2 and SIV in MT-4 cells and the results showed that the free ligands in fact exhibited a non-selective toxicity. They showed activity against the virus-infected cells but they were also cytotoxic against MT-4 cells with, as a main consequence, poor values in the selectivity index [SI<10 for most of them; only **11** presented a promising activity against HIV-1 (SI=23) and **3** against HIV-2 (SI=14)]. Owing to the known photochemical properties of the unmetallated porphyrins, their non-selective cytotoxic activity might be related to the light-dependent activation of the porphyrin chromophore (Bonnett, 1995). In the case of the two hydrophobic series **12/12-M** and **13/13-M**, all compounds, metallated or not, presented a low cytotoxicity and a negligible antiretroviral activity (data not shown).

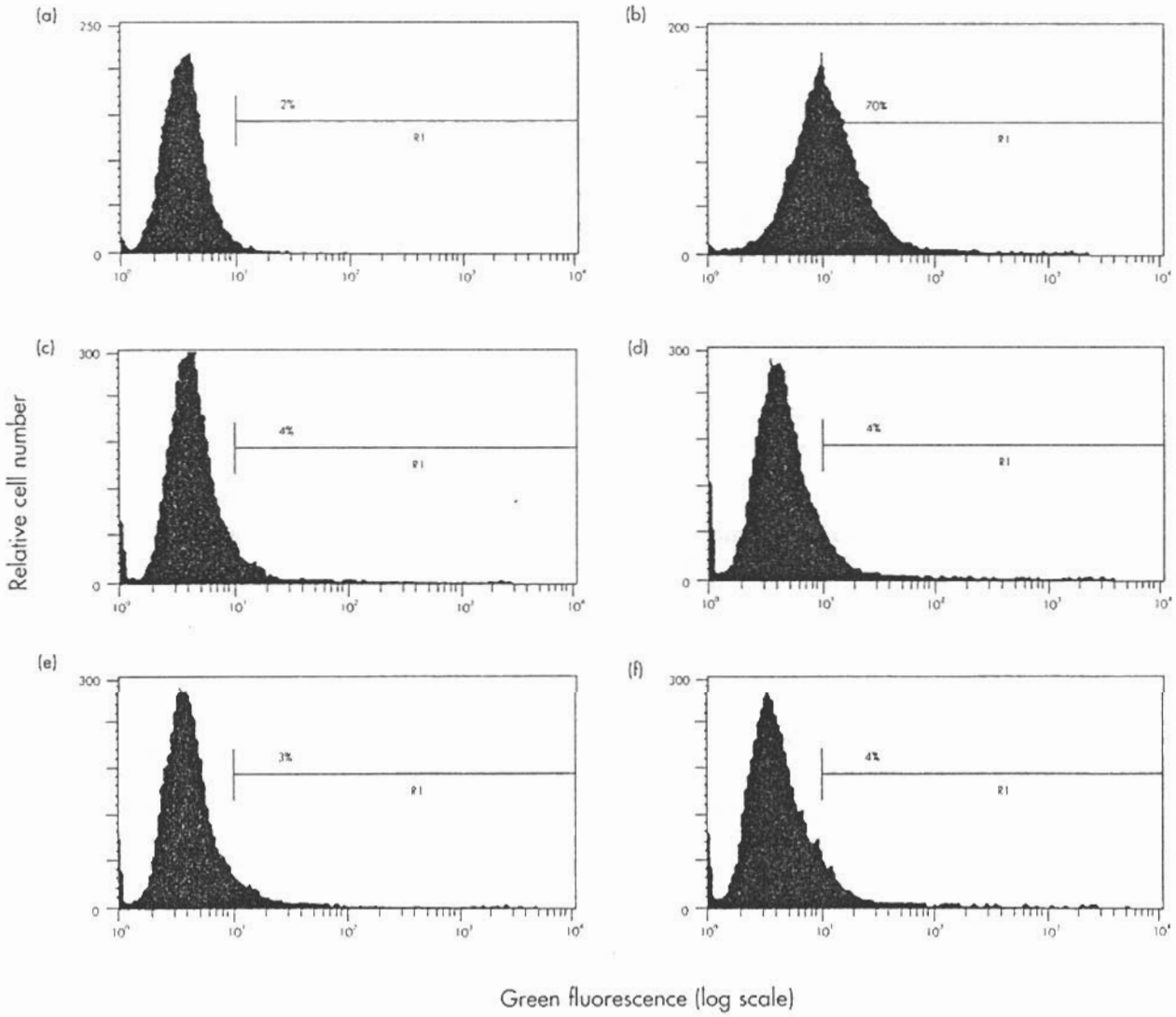
Unlike the free ligands and as expected, the metallated

derivatives presented generally low cytotoxicity associated with a selective toxicity against the different viruses (see SI data, Table 3). Moreover, this activity depended in part on the nature of the central metal: usually manganese derivatives were less active than the iron ones (except for **8-Mn**/**8-Fe**). The behaviour of nickel derivatives was intermediate: note the high and specific activity of **9-Ni** and **11-Ni** against HIV-1. Concerning the weak acids **4-M** and **9-M** (M indicates Mn, Fe or Ni), they were all active against HIV-1 but it was not possible to correlate the slight differences in response intensities with physical parameters like pK_a or partition coefficient (Table 2).

HIV-1 was more sensitive to most of the metalloporphyrins than HIV-2 (except for **3-Ni** and, to a lesser extent, **6-Fe**). SI was >10 in 13 cases for HIV-1, and in six cases for HIV-2. The selectivity against HIV-1 was especially marked for the three compounds **9-Mn**, **9-Fe** and **9-Ni** (SI>10) that had no noticeable activity against HIV-2.

Some compounds were evaluated and found to be active against SIV (e.g. metallated derivatives of **5**, **6** and **11**). The optimum activity was observed for **5-Fe** (SI>100).

Figure 1. Inhibition by anionic porphyrins of the binding of anti-gp120 MAb to HUT-78/HIV-1-infected cells.



HIV-1-infected HUT-78 cells were incubated with RaM-IgG(Fab')$_2$-FITC only (a), anti-gp120 MAb (9284) and RaM-IgG(Fab')$_2$-FITC (b), or anti-gp120 MAb (9284) and RaM-IgG(Fab')$_2$-FITC together with compound **5** (c), compound **5-Fe** (d), compound **11** (e) or compound **11-Fe** (f) at 5 μg mL^{-1}. The values indicated in each histogram represent the percentage of fluorescent-positive cells in region 1 (R1).

Other antiviral assays. In addition to the anti-HIV-1, HIV-2 and SIV assays, we performed several other antiviral assays with the most active derivatives in two families (**5**, **5-Mn**, **5-Fe** and **11**, **11-Fe**, **11-Ni**). From these tests, it is clear that **5-Fe** (and not **5** or **5-Mn**, data not shown) has some activity against HSV-1, HSV-2, Sindbis virus and Semliki forest virus, and also against VZV and CMV at a concentration well below the cytotoxicity threshold. This makes **5-Fe** an interesting antiviral agent with broad-spectrum antiviral activity. Compound **11** and its iron and nickel derivatives also exhibited a selective activity against CMV (Table 4).

Viral protein gp120 or CD4 receptor as possible targets of the anionic porphyrins. In our experiments we used either (i) a persistently HIV-1-infected $CD4^+$ cell line (HUT-78/HIV-1) and a specific anti-gp120 MAb recognizing the gp120 epitope responsible for syncytium (giant cell) formation or (ii) MT-4 cells and a specific anti-Leu3a MAb recognizing the CD4 cell receptor for HIV. As shown in Fig. 1, the four tested porphyrins, metallated (**5-Fe**, **11-Fe**) or not (**5**, **11**), had a marked inhibitory effect on the binding of the anti-gp120 MAb to the viral envelope gp120, even at a concentration as low as 5 $\mu g\ mL^{-1}$. In these conditions, inhibition was in the range 90–100%. On the other hand, the same compounds were unable (slight effect of **5** and **11-Fe**) to prevent the binding of anti-Leu3a MAb to the CD4 receptor of MT-4 cells, even at a concentration of 25 $\mu g\ mL^{-1}$ (data not shown). Thus compounds **5**, **5-Fe**, **11** and **11-Fe** do not interact with the cellular receptor (CD4) for HIV but directly interact with the viral glycoprotein gp120 that is involved in HIV binding to the cells and syncytium formation.

Inhibitory effect against giant cell formation. The most active anti-HIV compounds were tested for their inhibitory effect on HIV-induced giant cell formation (Table 5). The polyanion dextran sulphate MW 5000 was included as a positive control. The results obtained correlated with the results from the anti-HIV activity assay: the most active anti-HIV agents were also the most active in inhibiting giant cell formation between HUT-78/HIV-1 or HUT-78/HIV-2 cells and uninfected MOLT-4 cells. All compounds were more active against HIV-1-induced giant cell formation than against HIV-2-induced giant cell formation, compounds **1-Fe**, **4-Fe** and **11** being the most active.

Discussion

From inspection of the data collected for all the studied porphyrins, metallated or not (the main results are indicated in Tables 2 and 3), it can be concluded that:

(i) The labile ester series (**12** and **13** derivatives) did not present any particular advantages in these cellular tests, probably because of the absence of adequate hydrolysis (chemically or enzymatically) under the *in vitro* experimental conditions. However, they retain potential interest in the case of *in vivo* trials where the masked anionic charge could allow an improved absorption and where endogenous esterases could increase the hydrolysis rate to release the corresponding anionic derivative.

(ii) Derivatives in the **3** and **8** series (free sulphonamides) did not present noticeable activity (except for **8-Mn**, SI>12) whereas the corresponding acetylated derivatives (series **4** and **9**) appeared much more active with a marked selectivity against HIV-1 (SI ranging from 12 to 70). No suitable correlations could be found in this series of weakly acidic derivatives between antiviral activities and pK_a values, partition coefficient or nature of the chelated metal. In these series, compounds **4-Fe** and **9-Ni** appeared to be the most promising derivatives. Since acetylation is a usual way of metabolism, the presently unactive sulphonamides described above should merit evaluation *in vivo* for a possible conversion to their active forms.

(iii) Among the truly anionic derivatives at physiological pH, compounds **5-Fe** and **11-Fe** proved effective at concentrations well below their cytostatic concentrations with pronounced effects against HIV-1 (SI>25 for **5-Fe**, >180 for **11-Fe**), HIV-2 (SI>25 for **5-Fe**, >22.5 for **11-Fe**) and SIV (SI>100 for **5-Fe**, >7.5 for **11-Fe**). Since these activities were also found against some other viruses (e.g. CMV and HSV for **5-Fe** and **11-Fe**, VZV, Sindbis virus and Semliki forest virus for **5-Fe**), these two latter compounds appeared to be promising derivatives with a large spectrum of activities.

What is the possible target of these anionic metalloporphyrins? Even if some differences appeared in the activity of the related porphyrins metallated with various transition metals, electrostatic interaction between the anionic charges of the porphyrin ligand and viral envelope proteins or lymphocyte receptors seems to be the first mechanism to be considered. Following this hypothesis, the target might be either the viral protein gp120 or the CD4 receptor of lymphocytes. Results in Fig. 1 indicate that selected porphyrin derivatives inhibited the binding to HIV-infected cells of MAb 9284, specific for the V3 loop of the envelope glycoprotein gp120, and that this effect did not depend on the insertion of metal into the porphyrin. In addition, since the gp120 epitope of HUT-78/HIV-1 cells is required for cell fusion (syncytium formation), the inhibitory effect of anionic porphyrins against giant cell formation was studied and shown to be close to that of dextran sulphate as the polyanion reference (Table 5). These observations confirm the previous work of Neurath *et al.* (1992) showing that porphyrins containing anionic groups may interact with and block some of the

positively charged sites of the V3 loop. In this respect, compound **5-Fe**, bearing eight sulphonate groups at the periphery of the porphyrin ligand, is one of the most active derivatives of the metalloporphyrin series.

Within the same series, the antiretroviral effect of the iron derivative was higher than the manganese one (see couples **1-Mn/1-Fe**, **4-Mn/4-Fe**, **5-Mn/5-Fe** and, to a lower extent, **9-Mn/9-Fe**). This might correspond to an additional effect of these redox active metal complexes responsible for oxidative damage at the site of interaction on gp120, after a reductive activation process involving molecular oxygen and an electron source, as previously observed for the formation of 'activated' bleomycin, a naturally occurring DNA cleaver (Pratviel *et al.*, 1995). Alternatively, a possible coordination of an amino acid residue of the viral protein gp120 could also contribute to the dependence of the activity on the nature of the central metal ion. Finally, it must be noted that nickel derivatives **4-Ni**, **9-Ni** and **11-Ni** were also fairly active. This activity is probably not related to a redox activity of the metal centre, but we have to keep in mind that nickel porphyrin derivatives are not easily oxidized but they can accept axial ligands (Antipas & Gouterman, 1983).

In conclusion, a series of various water-soluble polyanionic porphyrins (polysulphonated and polycarboxylated ones) were prepared. Aimed at bioavailability modulation, acetylsulphonamido derivatives endowed with weak acidity properties (deprotonation gives the corresponding anionic derivatives in a pH range 4.5–8.5) were also synthesized. Since anionic metalloporphyrins are charged molecules and may not be easily taken up by cells, a second series of compounds with the anionic charge transiently masked by esterification (acetoxymethyl- and pivaloyloxymethylesters) was prepared. All these compounds were evaluated against HIV-1, HIV-2, SIV and some of them against CMV, HSV, VZV, Sindbis virus and Semliki forest virus. Free porphyrin ligands were more cytotoxic than the corresponding metallated derivatives. The latter compounds exhibited a selective activity against different viruses, and iron complexes were more active than the corresponding manganese derivatives. Nickel complexes were also active against HIV-1. Among all the different anionic metalloporphyrins tested, the sulphonated iron complex **5-Fe** had a good SI and exhibited a broad-spectrum antiviral activity, not only against HIV-1, HIV-2 and SIV, but also against HSV-1, HSV-2, Sindbis virus, Semliki forest virus and VZV. Among the sulphonamide derivatives, compounds **4-Fe** and **9-Ni** were the most promising. The mode of action of the polyanionic metalloporphyrins is comparable to that of dextran sulphate. They mainly interact with the viral HIV protein gp120 and not with the CD4 cellular receptor. Because of their broad spectrum activity, some iron porphyrin derivatives should be further considered for *in vivo* evaluation.

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