Antipoliovirus Structure−Activity Relationships of Some Aporphine Alkaloids

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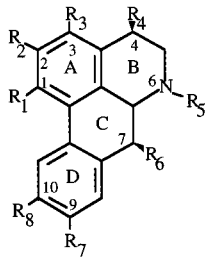
A series of 18 aporphinoids have been tested in vitro against human poliovirus. The aporphines (+)-glaucine fumarate (**1**), (+)-*N*-methyllaurotetanine (**4**), (+)-isoboldine (**7**), and (−)-nuciferine, HCl (**10**) were found to be active with selectivity indices >14. The nature of the 1,2-substituents of the isoquinoline moiety appeared to be critical for antipoliovirus activity. An SAR study demonstrated the importance of a methoxyl group at C-2 on the tetrahydroisoquinoline ring for the induction of antipoliovirus activity. Molecular modeling of some compounds in this series revealed the close similarities between the three-dimensional conformational features of the inactive 1,2-substituted derivatives (+)-boldine (**6**) and (+)-laurolitsine (**5**) with derivatives containing the 1,2-(methylenedioxy) moiety, which were generally found to be inactive as exemplified by (+)-cassythicine (**9**).

Alkaloids are a rich source of bioactive products, and isoquinoline compounds exhibit a wide range of biological activities.[1] Among these isoquinoline derivatives, aporphinoids that are mainly encountered in some archaic botanical families[2] appear to be increasingly investigated for biological activity. For instance, recent experiments on the aporphinoid series have focused on promising antiplatelet and vasorelaxing effects.[3]

Numerous alkaloids have also been tested for activity against various viruses[4] and viral enzymes. Isoquinolines demonstrating good in vitro activities include benzophenanthridines (e.g., fagaronine, nitidine) and emetine derivatives (e.g., psychotrine) in reverse transcriptase inhibition assays[5−7] or Amaryllidaceae alkaloids against several RNA-viruses (e.g., lycorine, pancratistatin).[8,9] Consistent anti-HIV results obtained in vitro with the benzylisoquinoline papaverine[10] and the bisnaphthylisoquinoline michellamine B[11] led to preclinical drug development.[12,13]

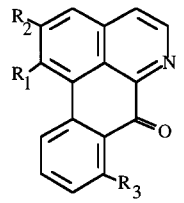
The antiviral potency of isoquinolines has been demonstrated but has yet to be thoroughly investigated. As various isoquinoline alkaloids isolated from plants were available, we screened them for antiviral activity against DNA and RNA viruses. Results obtained for an aporphinoid series against the DNA-virus, Herpes simplex virus hominis type 1 (HSV-1), have been previously reported.[14] Herein, we report the activity of 18 of these compounds against the poliovirus, an RNA-picornaviridae. Structure−activity relationships have been investigated, and molecular modeling studies for some of these aporphines have been performed in an attempt to better comprehend their activity. The alkaloids tested were as follows: tetrahydroisoquinoline derivatives (H-6a-α (**1**−**10**) and H-6a-*β* (**11**−**15**)) and oxoaporphines (**16**−**18**).

APORPHINES AND NORAPORPHINES



	6a*	R1	R2	R3	R4	R5	R6	R7	R8
1	S	OCH_3	OCH_3	H	H	CH_3	H	OCH_3	OCH_3
2	S	OCH_3	OCH_3	H	H	$COCH_3$	H	OCH_3	OCH_3
3	S	OCH_3	OCH_3	H	H	H	H	OH	OCH_3
4	S	OCH_3	OCH_3	H	H	CH_3	H	OH	OCH_3
5	S	OCH_3	OH	H	H	H	H	OH	OCH_3
6	S	OCH_3	OH	H	H	CH_3	H	OH	OCH_3
7	S	OH	OCH_3	H	H	CH_3	H	OH	OCH_3
8	S	O-CH_2-O		H	H	H	H	OH	OCH_3
9	S	O-CH_2-O		H	H	CH_3	H	OH	OCH_3
10	R	OCH_3	OCH_3	H	H	CH_3	H	H	H
11	S	O-CH_2-O		OCH_3	H	CH_3	OH	H	H
12	S	O-CH_2-O		H	H	CH_3	OH	OCH_3	H
13	S	O-CH_2-O		H	H	CH_3	OCH_3	OCH_3	H
14	S	O-CH_2-O		H	H	H	OCH_3	H	H
15	S	O-CH_2-O		H	OH	CH_3	OCH_3	H	H

OXOAPORPHINES



	R1	R2	R3
16	O-CH_2-O		H
17	OCH_3	OCH_3	H
18	O-CH_2-O		OCH_3

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Table 1. In Vitro Antiviral Effect against Poliovirus of 18 Aporphinoid Alkaloids

alkaloid	cytotoxicity CC_{50}[b]	CPE inhibition ED_{50}[c]	CPE inhibition SI[d]	tested concentrations MTC[e]	tested concentrations MTC/2[e]	yield reduction[a] 0.1[f]	yield reduction[a] 0.01[f]	yield reduction[a] 0.001[f]
glaucine fumarate (**1**)	142	9	15.8	62		1	1.5	3.0
					31	0.5	1.0	1.5
N-acetylnorglaucine (**2**)	342	50	6.8	250		1.5	1.5	1.5
					125	0.5	0.5	1.0
laurotetanine, HCl (**3**)	165	31	5.3	62		1.0	1.5	1.5
					31	0.5	0.5	1.0
N-methyllaurotetanine (**4**)	250	15	16.7	125		1.0	1.5	2.5
					62	0.5	1.0	1.0
laurolitsine (**5**)	95	62	1.5	62		−	−	−
boldine (**6**)	250	125	2.0	125		−	−	−
isoboldine, HCl (**7**)	217	15	14.5	125		1.5	1.5	2.5
					62	1	1	1.5
actinodaphnine (**8**)	107	−	−	95		−	−	−
cassythicine (**9**)	47	−	−	31		−	−	−
nuciferine, HCl (**10**)	435	26	16.7	125		1.0	1.0	2.0
					62	0.5	0.5	0.5
guatterine (**11**)	107	62	1.7	62		−	−	−
oliveridine (**12**)	7	−	−	5		−	−	−
oliverine, HCl (**13**)	9	−	−	7		−	−	−
pachypodantine, HCl (**14**)	25	−	−	20		−	−	−
pachystaudine (**15**)	68	31	2.2	62		0	0.5	0.5
liriodenine (**16**)	48		−	31		−	−	−
lysicamine (**17**)	500	−	−	250		−	−	−
oxostephanine (**18**)	28	−	−	15		−	−	−

[a] The yield reduction was expressed as the log (±0.5) reduction of virus titer by comparison with controls. Assays were performed at MTC and MTC/2. [b] CC_{50} = 50% cytotoxic concentration (μM). [c] ED_{50} = effective concentration (μM) required to inhibit cytopathic effect by 50%, at an MOI of 0.001. [d] SI = selectivity index CC_{50}/ED_{50}. [e] MTC = maximum tolerated concentration (μM). [f] 0.1; 0.01; 0.001 = multiplicities of infection (MOI), see Experimental Section. (−) = not done.

Results and Discussion

A series of 10 aporphines (**1**, **4**, **6**, **7**, **9**−**13**, and **15**) and five noraporphines (**2**, **3**, **5**, **8**, and **14**) along with three oxoaporphines (**16**−**18**) were tested in vitro against poliovirus. A microscope examination of the cytopathic effect (CPE) compared to controls revealed the antipoliovirus activity. Values obtained for cytotoxicity on Vero cells (CC_{50}), 50% CPE inhibition (ED_{50}), and selectivity indices (SI) are given in Table 1. The log reduction of virus titers determined after multiple rounds of replication has also been attempted for the maximum tolerated concentration, MTC (concentration at which no cellular alteration was microscopically detectable), and MTC/2 in order to specify the activity of the most active compounds.

In this experiment, compounds **1**, **4**, **7**, and **10** presenting a SI > 14 with a yield reduction of the viral titer ≥ 2 $\log_{10}$ when poliovirus was inoculated at the viral multiplicity of infection (MOI) of 0.001 are considered as active. Compounds with a SI < 5 and a yield reduction titer <1 $\log_{10}$ are considered as inactive. Thus, the noraporphines **2** and **3** may be considered as weakly active natural products.

When comparing the anti-HSV-1[14] and the antipoliovirus activities of this aporphinoid series, a contrasting viral susceptibility profile is apparent; the active compounds against poliovirus being inactive against HSV-1 and vice versa. This observation attests to a specific antiviral mechanism. A preliminary examination of the mechanism of action of one of the most active compounds, glaucine fumarate (**1**), has, moreover, shown that it acts at the postentry level in the poliovirus replicative cycle since, in this experiment, the compound was active even when added 60 min postinfection. The activity of **1** was further confirmed by a 2 $\log_{10}$ yield reduction in virus titer, inoculated at MOI = 1, after a single cycle of replication, whereas neither preincubation with virus nor cells had induced any viral yield reduction, indicating that the compound does not act via interaction with viral or cell surface components.

Among the diverse compounds with an isoquinoline skeleton that have been tested against poliovirus and related picornaviridae, the benzophenanthridine, nitidine chlorydrate,[15] the terpenic isoquinoline alkaloid, emetine,[16] the Amaryllidaceae alkaloid, lycorine,[9] and the phenanthroquinolizidine, cryptoleurine,[16] have all shown an in vitro activity. As many of the isoquinoline derivatives tested here were found to be inactive, the isoquinoline skeleton is evidently not sufficient to ensure an antipoliovirus effect. Moreover, the intact aporphinoid skeleton seems to be of importance for antipoliovirus activity since the (1a−11a) seco derivative of the active isoboldine (**7**) (i.e., the benzylisoquinoline reticuline) was 5-fold less toxic and nearly inactive (CC_{50} = 1150 μM, ED_{50} = 500 μM) in our testing (data not shown).

The three oxoaporphines tested in this study were found to be inactive against poliovirus. The introduction of the oxofunction and/or aromatization of the B-ring appear to negate the antiviral effect against poliovirus since lysicamine (**17**), the noroxodehydro derivative of **10**, has a comparable toxicity on host cells but no antipoliovirus activity. However, the limited number of compounds tested here, and the undefined roles of the other molecular substituents present, do not allow any definitive and precise conclusions to be made. The *N*-methylation of **3**, **5**, and **8** to give the noraporphines **4**, **6**, and **9**, respectively, and the *N*-acetylation of **1**, resulted in an insignificant effect on host cell toxicity and antipoliovirus activity, although the noraporphine (+)-hernovine has been shown to have more potent cytotoxic activities than its *N*-methyl analogue.[17] This

suggested that *N*-substitution has a limited effect on antipoliovirus activity. In contrast, the antipoliovirus activity of these alkaloids appeared to be highly sensitive to 1- and 2-isoquinoline substitution. Compounds bearing a 1,2-methylene dioxide function are inactive. The negative role of this group can be illustrated when comparing **7**, one of the most active aporphines, to its totally inactive methylenedioxy analogue, **9**. Moreover, the 1,2-(methylenedioxy) substitution largely contributes to the host cell cytotoxicity of aporphines *sensu stricto*, as exemplified by the 5-fold toxic effect of **9** over that of **4**, **6**, and **7** and by the 10-fold toxic effect of liriodenine (**16**) over that of **17**. Such a methylenedioxy function has also been recognized to contribute to the cytotoxicity of dicentrine methyne, an isoquinoline phenanthrene.[18]

Although the placement of the functional groups in ring A might be of greater importance than their constitution in the case of the cytotoxic benzophenanthridines alkaloids,[19] the nature of the substituents appears to be of crucial importance in this study. Laurotetanine (**3**), with a 2-OCH_3 substitution, exhibits an antiviral activity, whereas its 2-OH counterpart laurolitsine (**5**) is inactive. Moreover, the mere transposition of the 1- and 2-substituents of isoboldine (**7**), to correspond to those of boldine (**6**), led to a loss of activity. On the other hand, the replacement of the 1-OH group of **7** by a OCH_3 moiety to give *N*-methyllaurotetanine (**4**) has little effect on the antipoliovirus activity, and all the 1,2-dimethoxyaporphines screened here (**1**−**4** and **10**) were found to be active. These data indicate that substitution at position 2 is a crucial element in the interaction process between these alkaloids and their molecular target. To determine more precisely a pharmacophoric feature required for antiviral activity, a computation of the conformational stability of the 1- and 2-bonded isoquinoline substituents was performed.

Due to the flexibility of the tetrahydroisoquinoline ring, each of these compounds may exist in four different low-energy conformations, in which the NCH_3 group can occupy either a pseudoaxial or a pseudoequatorial position and, moreover, adopt either a pseudo-trans or a pseudo-cis position relative to the C-6a−H bond. According to AM1 calculations, in the H-6a-α-series of aporphines, the energetically favored conformations are those in which the NCH_3 exhibit a pseudo-trans/pseudoaxial position in their free base form and a pseudo-trans/pseudoequatorial position in their protonated form. This latter result fits the X-ray crystal structure of isoboldine hydrobromide.[20] In the absence of information about the interaction between substrate and target, the conformational studies were performed both on the free base and on protonated forms in their lowest energy conformation. The conformational analysis was directed at predicting the preferred states about τ_1 ($C_2-C_1-O_1-X_1$) and τ_2 ($C_3-C_2-O_2-X_2$) dihedral angles for isoboldine (**7**) and *N*-methyllaurotetanine (**4**), both strong antipoliovirus compounds, and for the inactive boldine (**6**) (X = H or CH_3). The lowest energy conformation for these three compounds as well as for cassythicine (**9**), in their protonated forms, as calculated by the AM1 Hamiltonian, is shown in Figure 1. Very similar profiles have been obtained with the free base forms of these compounds (data not shown). Thus, all conformational interpretations concerning the protonated forms would also apply to the free base forms.

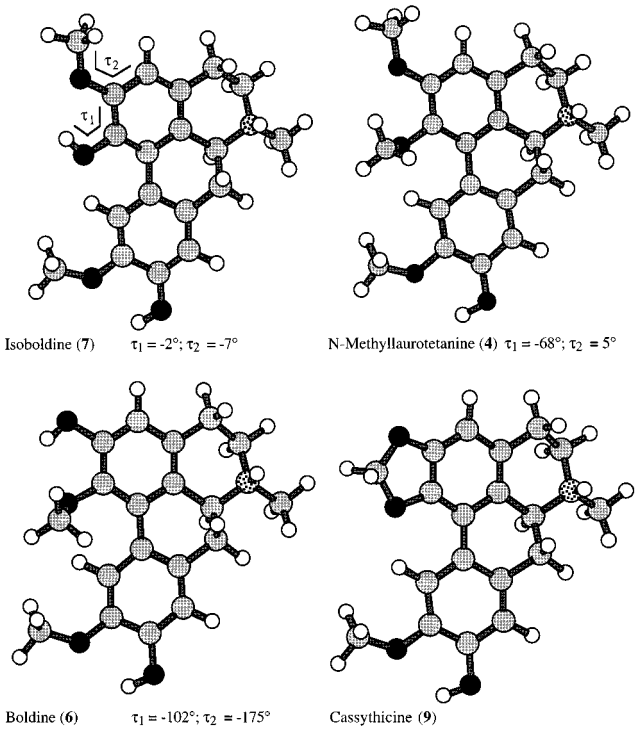

Figure 1. Molecular models of **7**, **4**, **6**, and **9** (protonated forms) in their lowest energy conformation. Values of the τ_1 and τ_2 dihedral angles (as shown on structure **7**) corresponding to the AM1-calculated absolute minimum are specified.

The conformational energy map of **7** (data not shown) displays four relative minima, actually corresponding to a zone where the two substituents are nearly coplanar to the benzene ring. The global minimum-energy conformation is located in this zone, and the torsion angles issuing from the AM1 minimization are $\tau_1 = -2°$ and $\tau_2 = -7°$ for this compound. A similar conformational profile is obtained for the active analogue **4**, although the AM1 calculated absolute minimum is displaced near $\tau_1 = -68°$ and $\tau_2 = 5°$ due to steric hindrance imposed by the 1-methoxyl group. The inactive boldine (**6**) displays four equal minimum energy regions that overlap with the four minima of **4**. These regions correspond to a secondary energy minimum (C_1 conformation at $\tau_1 = -40°$ and $\tau_2 = 2°$). Compound **6** is, in other respects, characterized by a distinct zone, not found with the 2-methoxy compounds **4** and **7**, in which the absolute minimum energy is located (C_2 conformation at $\tau_1 = -102°$, $\tau_2 = -175°$). This calculated conformation results from a twist of the 2-OH bond (τ_2) to about 180° with regard to the local minimum C_1 and corresponds to a stabilizing intramolecular interaction between the 2-hydroxyl and the 1-methoxyl groups. According to AM1 calculations, the transition from the C_2 to the C_1 conformation needs energy higher than 5 kcal/mol. This energy barrier could be sufficient to prevent the mobilization of the inactive compounds **5** or **6** at their site of action, either directly on indirectly. As a result, their structural features might mimic those of the methylenedioxy derivatives and, consequently, be inactive against poliovirus. As can be seen in Figure 1, the active 2-methoxy derivatives **4** and **7** are mainly

distinguishable from the inactive **6** and **9** analogues and to a certain extent from other methylenedioxy derivatives, through their overall molecular shape, and through the electronic accessibility in the 1,2-region of the molecule. The out-of-plane orientation of the electron lone pairs of the 1,2-oxygen atoms of the inactive compounds might be inconsistent with a possible electrostatic or H-bonding interaction between the ligand and its target site. The importance for activity of a 2-methoxyl substituent could also be related to a steric effect involved in a "lock and key" system.

However, additional compounds have to be studied in order to confirm these observations. In addition, the molecular target involved in the interaction process of aporphinoids with poliovirus needs to be identified before the exact role played by electrostatic interactions and/or molecular shape complementarity may be elucidated.

Experimental Section

General Biological Procedures. Cells, culture media, alkaloid solubilization, and the method used for the evaluation of cytotoxicity are given in ref 14.

Virus. A virus stock of poliomyelitis virus type 2 vaccinal strain Sabin II (poliovirus) was prepared by infecting Vero cells at a low multiplicity. Incubation was continued until complete cellular destruction (about 1 day) was observed. The culture was then frozen and thawed three times. The preparation was clarified by centrifugation at low speed to remove cell debris. Virus titration was performed by limiting dilution, using six wells of a 96-well Nunc microplate per dilution. The virus titer was estimated from cytopathogenicity and expressed as 50% tissue culture infectious dose.mL^{-1} ($TCID_{50}$/mL). The titer of the currently used stock was determined to be $2.0 \times 10^{4.7}$ $TCID_{50}$ mL^{-1}.

Antiviral Testing. Stock solutions of alkaloids (10 mM) were prepared with DMSO (5%) and distilled water (95%) and kept in aliquots stored at −20 °C. Dilutions of these alkaloid solutions in culture medium, from MTC (maximal tolerated concentration) to MTC/5 were added to confluent 1-day-old monolayer cultures of Vero cells grown in microtiter tissue culture plates, just before inoculation at a low multiplicity of infection: MOI = 0.001 $TCID_{50}$ per cell. Toxicity controls, cell controls, and virus controls were performed simultaneously. The assay of each drug concentration was carried out in sextuplicate. Plates were incubated at 37 °C for 32 h, and then the monolayers were microscopically observed for cytopathic effect. The concentration of the alkaloid that inhibited the viral cytopathic effect by 50% (compared to the controls), using a quantitative procedure, was expressed as the 50% effective dose (ED_{50}). To specify the antiviral activity of compounds **1**−**4**, **7**, **10**, and **15**, the same experiment was performed at three multiplicities of infection 0.1; 0.01 and 0.001 $TCID_{50}$ per cell with alkaloid concentrations corresponding to MTC and MTC/2. After multiple replicative cycles (incubation for 32 h), the plates were frozen and thawed three times to obtain a maximum release of virions from cells. The contents of the six identical wells were harvested, mixed, and clarified by low-speed centrifugation, and virus titrations were performed on the supernatant fluids by the limiting dilution method. The antiviral activity of each alkaloid was determined as the reduction factor (log_{10}) of the viral titer in comparison with untreated controls. Results were expressed as the mean of three separate experiments.

Study of the Mode of Action of Glaucine fumarate (1). In all experiments, **1** was tested at its MTC, 62.5 μM.

Virus Inactivation. Equal volumes of poliovirus stock suspension (500 $\mu L \times 2.0 \times 10^{4.7}$ $TCID_{50}$ mL^{-1}) and a solution of **1** were mixed and incubated for 2 h at 37 °C. Thereafter, the mixture was diluted 10-fold serially, and infectious titers were compared to those of controls.

Yield Reduction after a Single Cycle of Replication. Vero cell monolayers in four-well culture plates were infected with poliovirus at a multiplicity of infection of about 1. After 60 min at 37 °C, the unadsorbed virus was removed, and the monolayers were washed twice with MEM then refed with MEM containing **1**. After incubation for 8 h, which corresponds to about one cycle of replication,[21] the supernatant virus titers were determined by limiting dilution and compared to that of controls, as decribed above.

Culture Pretreatment. Vero cell monolayers were pretreated with **1** for 24 h at 37 °C. After washing with MEM, the cells were exposed to poliovirus at an MOI of about 1. Then, the experiment was performed as described above except that no drug was added to the medium.

Molecular Modeling Techniques. Initial ligand structures were generated interactively using the Molecular Advanced Design software package (MAD).[22] Molecular mechanic techniques, MM2 force field,[23] were used to determine the preferred conformations and to perform conformational analysis. The rotatable bonds were explored using a stepwise increment at 20° resolution. MM2-minimized structures were then reoptimized using the semiempirical quantum mechanical AM1 method[24] included in the MOPAC v. 6.0 package,[25] using the PRECISE keyword. All simulations were computed on a Silicon Graphics Indigo R3000 workstation.

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