ELSEVIER

Eur. J. Med. Chem. 37 (2002) 3–10

EUROPEAN JOURNAL OF MEDICINAL CHEMISTRY

Original article

Pyrrolo[1,2-*f*]phenanthridines and related non-rigid analogues as antiviral agents☆

Anna Maria Almerico [a,*], Francesco Mingoia [b], Patrizia Diana [a], Paola Barraja [a], Alessandra Montalbano [a], Antonino Lauria [a], Roberta Loddo [c], Laura Sanna [c], Donatella Delpiano [c], Maria Giovanna Setzu [c], Chiara Musiu [c]

[a] *Dipartimento Farmacochimico, Tossicologico e Biologico, Università degli Studi, Via Archirafi 32-90123 Palermo, Italy*
[b] *Istituto di Chimica e Tecnologia dei Prodotti Naturali-CNR, Via Ugo la Malfa 153-90146 Palermo, Italy*
[c] *Dipartimento di Biologia Sperimentale, Sezione di Microbiologia, Università di Cagliari, Cittadella Universitaria, SS 554-09042 Monserrato Cagliari, Italy*

Received 3 March 2001; received in revised form 28 September 2001; accepted 3 October 2001

Abstract

The pyrrolo[1,2-*f*]phenanthridines **8**–**22** and the corresponding non-rigid analogues **23**–**41** were synthesised and their ability to inhibit the replication of HIV-1 was tested. Only the polycyclic derivatives **10**, **11**, and **13** showed a weak *anti*-HIV activity, whereas several pyrrolo-phenanthridines (**8**, **10**, **16**–**18**) were found to stimulate the multiplication of MT-4 cells at low concentrations. Derivative **10** demonstrated to possess the unique property of stimulating the multiplication of lymphocytes joined to HIV inhibition.

Keywords: pyrrolo[1,2-*f*]phenanthridines (PPHs); non-rigid analogues; *anti*-HIV activity

1. Introduction

In the course of our studies on polycyclic nitrogen heterocycles we have been exploring synthetic approaches to the pyrrolo[1,2-*f*]phenanthridine ring system (PPH) to prepare derivatives with potential anti-neoplastic activity. In fact PPHs are structurally related to ethidium bromide and to 9-acridinylmethanesulfonanilide derivatives (AMSA), compounds capable of intercalating into double-stranded DNA. With the aim of approaching SAR studies on PPH derivatives, we have proposed synthetic methods [1–4] that allow functionalisation of the phenanthridine moiety with substituents such as amino and/or methoxy groups suitable for the interaction with DNA base pairs, and in preliminary biological screening tests, several PPHs have shown interesting anti-proliferative activity against Friend erythroleukemia cells (FLC) and the multi-drug resistant cell lines (DRTL, derived from the former through exposure to increasing doses of daunorubicin) with IC_{50} in the range 5–50 μM [5].

As an extension of these studies we became interested in testing our compounds for anti-replicative activity against viruses, in particular human immunodeficiency virus (HIV), since the incorporation of the pyrrole moiety has already led to several classes of non-nucleoside reverse transcriptase inhibitors (NNRTI) such as derivatives **1**–**5** [6–11] (Fig. 1). The NNRTIs bind to an allosteric site on the enzyme at a close distance from the active site and generally are effective without toxic effects, even if the rapid emergence of NNRTI-resistant viral strains has greatly limited the clinical efficacy of these compounds. Biological activity of these derivatives seems to be associated to their capability to

☆ Presented in part at XIV Convegno Nazionale Divisione di Chimica Farmaceutica della S.C.I., Salsomaggiore Terme-Parma, September 1998, Abstr p. 182.

* Correspondence and reprints.
E-mail address: almerico@unipa.it (A.M. Almerico).

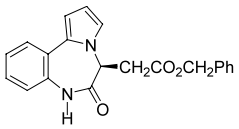
0223-5234/02/$ - see front matter
PII: S0223-5234(01)01289-2

assume a 'butterfly-like' conformation as observed for example in the case of nevirapine (**6**) and TIBO R86183 (**7**) (Fig. 2) [10]. On the basis of these premises, now we describe our results on in vitro *anti*-HIV-1 activity of PPHs and related non-rigid analogues.

2. Results and discussion

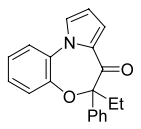
2.1. Chemistry

The synthesis of pyrrolo[1,2-*f*]phenanthridine derivatives **8**–**20** (Table 1) herein reported was accomplished, according to the procedures already described by us, by Pschorr-type cyclisation reaction of diazotised 2-(2-aminoaryl)-1-arylpyrroles [1], or by decomposition of 2-(3-azidoaryl)-1-arylpyrroles [5] or 1-(3-azidoaryl)-2-arylpyrroles [4] catalysed by trifluoromethane-sulfonic



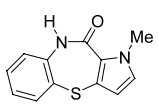
Pyrrolobenzodiazepine **1**

CC_{50} 10 μg/ml
EC_{50} 0.3 μg/ml



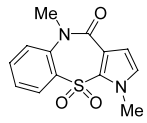
Pyrrolobenzoxazepine **2**

CC_{50} 10 μM
EC_{50} 0.3 μM



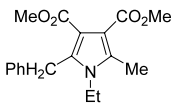
Pyrrolo[3,2-b]benzothiazepine **3**

CC_{50} >434 μM
EC_{50} 16 μM



Pyrrolo[2,3-b]benzothiazepine **4**

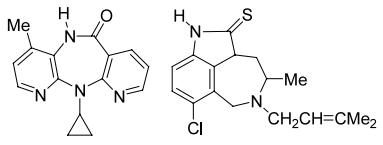
CC_{50} >362 μM
EC_{50} 14 μM



Highly substituted pyrrole **5**

CC_{50} 175 μM
EC_{50} 2.8 μM

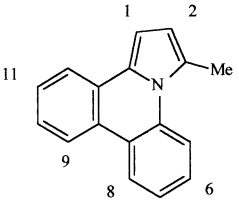
Fig. 1. Non-nucleoside reverse transcriptase inhibitors.



Nevirapine **6** TIBO R86183 **7**

Fig. 2. Compounds capable to assume a butterfly like conformation.

Table 1
Pyrrolo[1,2-*f*]phenanthridine derivatives



Compounds	Positions					
	1	**2**	**6**	**8**	**9**	**11**
8	Br	COOEt	H	H	H	H
9	H	COOEt	NH_2	H	H	H
10	H	COOEt	NH_2	H	H	OMe
11	H	COOEt	H	NH_2	OMe	H
12	H	COOEt	H	NH_2	H	OMe
13	H	COOEt	NH_2	H	OMe	H
14	H	COOEt	OMe	H	H	NH_2
15	H	COOEt	OMe	H	NH_2	H
16	Br	COMe	H	H	H	H
17	H	COMe	NH_2	H	H	H
18	H	COMe	NH_2	H	H	OMe
19	H	COMe	H	NH_2	H	OMe
20	H	COMe	NH_2	H	OMe	H
21	H	COOEt	N_3	H	H	OMe
22	H	COOEt	Cl	H	H	OMe

acid (TFMSA). The new compounds 6-azido-11-methoxy-pyrrolo[1,2-*f*]-phenanthridine (**21**) and 6-chloro-11-methoxy-pyrrolo[1,2-*f*]phenanthridine (**22**) were prepared from the corresponding 6-amino derivative **10** via its diazonium salt. The 1,2-diarylpyrroles **23**, **24**, **26**–**33**, **36**–**41** (Table 2), non-rigid analogues of the PPHs, were synthesised as already described by us [3,4]. The 5-(3-bromophenyl)pyrrole (**25**) was obtained from the corresponding 5-(3-aminophenyl)pyrrole (**24**), upon diazotisation with *iso*-amyl nitrite in acetonitrile, in the presence of copper(II) bromide [12]. 1-(3-Halophenyl)pyrroles **34** and **35** were prepared by a Paal–Knorr cyclisation reacting 1,4-diketone with appropriate 3-haloaniline.

2.2. Molecular modelling

The non-nucleoside binding site of HIV-1 RT is among the most extensively studied binding pockets. However, it seems that each NNRTI binds in a unique way. Recently, by using the butterfly shaped binding region described by Ding et al. [10], docking studies demonstrated that the Wing 2 region can accommodate heterocyclic rings with calculated molecular volumes in the range 260–275 $Å^3$ [11]; therefore we calculated the molecular volumes of all the PPHs and of all the selected non-rigid analogues in order to compare with those of the pyrrolo-containing NNRTIs and of

nevirapine and TIBO derivative **7**. The data reported in Table III show that the majority of pyrrole derivatives have volumes comparable with those of reference NNRTIs. The values of calculated accessible surface areas (ASA) resulted even more similar when comparing the polycondensed derivatives **8**–**22** and **7** or the highly substituted pyrrole **5**.

The molecular models of derivatives **8**–**41** and of the reference drugs **1**–**7** were constructed by using PIMMS V1.47 and VAMP V6.1 softwares [13], and optimised in vacuo by semi-empirical molecular orbital method PM3. The superimposition, in the conformation of minimum of energy, between nevirapine, derivative **7**, selected PPHs (planar) and the corresponding non-rigid analogues showed a good geometric fit between the structures (R.M.S. in the range 0.0270–0.0455) (Fig. 4).

All these data suggest that pyrrole containing derivatives could fit favourably into the NNRTI binding pocket.

2.3. Biology

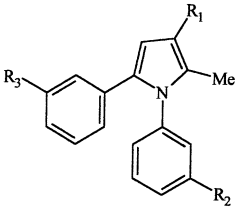
Test compounds were evaluated for anti-retroviral activity in MT-4 cells infected with HIV-1. Cytotoxicity in MT-4 cells was evaluated to determine whether test compounds were endowed with selective antiviral activity. AZT was used as reference compound.

Most of the compounds resulted inactive to protect MT-4 cells from the cytopathic effect induced by HIV-1. Only in the case of derivatives **10**, **11** and **13** a weak activity at concentrations not cytotoxic for MT-4 cell was found, with EC_{50} of 100, 120 and 60 μM, respectively.

However, despite the poor selectivity, it is noteworthy the ability of some compounds to stimulate the multiplication of MT-4 at concentrations between 50 and 200 μM in the case of compound **10** and at lower concentrations, between 1.5 and 6 μM, in the case of derivatives **8**, **16**, **17** and **18**, as reported in Fig. 3, where cell growth (OD) at different concentrations of compound is compared with an untreated control cell. Analogous results were obtained by measuring the cell density by staining with methylene blue [14]. Therefore, we suggest that the test compounds stimulated the cell multiplication. This is also confirmed by an increase in cell number of 20–35% with respect to untreated control, as determined by viable count with Trypan Blue Stain [15].

These derivatives were also tested in another T lymphocyte cell line, CEM, and compound **10** also in peripheral blood lymphocytes. In both cell lines it was possible to observe only a slight stimulation of multiplication (data not shown).

Table 2
1,2-Diarylpyrroles



Compounds	R_1	R_2	R_2
23	COOEt	NH_2	OMe
24	COOEt	OMe	NH_2
25	COOEt	OMe	Br
26	COOEt	H	NO_2
27	COOEt	H	NH_2
28	COOEt	H	N_3
29	COOEt	OMe	NO_2
30	COOEt	OMe	N_3
31	COOEt	NH_2	H
32	COOEt	N_3	H
33	COOEt	N_3	OMe
34	COOEt	Br	OMe
35	COOEt	Cl	OMe
36	COMe	H	NO_2
37	COMe	H	N_3
38	COMe	NH_2	H
39	COMe	N_3	H
40	COMe	NH_2	OMe
41	COMe	N_3	OMe

2.4. SAR studies

In Table 3, we also report the parameters chosen to define the lipophilicity of title compounds: i.e. the R_M (measured) and the log P (calculated) values. Actually, a good correlation was found between these two sets of data. In fact, regression analysis of the data for compounds **8**–**22** ($n = 14$) gives the following regression equation:

$$(\log P) = 6.044R_M + 2.162 \quad (1)$$

with $r = 0.904$; $r^2 = 0.816$ cv $= 0.761$; residual sum of squares $= 1.914$; predictive sum of squares $= 2.4888$.

In the case of the non-rigid analogues, it was impossible to find a good correlation, probably because the log P values, additively calculated for each substituent, do not account well for the difference between isomeric compounds. Therefore, in the case of PPHs, we could use these log P values to explore structure–activity relationship. A linear relationship was found between the calculated log P values and the observed antiviral activity (Eq. (2)).

$$EC_{50} = 95.58(\log P) - 207.7 \quad (2)$$

with $n = 14$; $r = 0.820$; $r^2 = 0.672$ cv $= 0.643$.

In this case the correlation is statistically less significant, but it is possible to evidence that less lipophilic

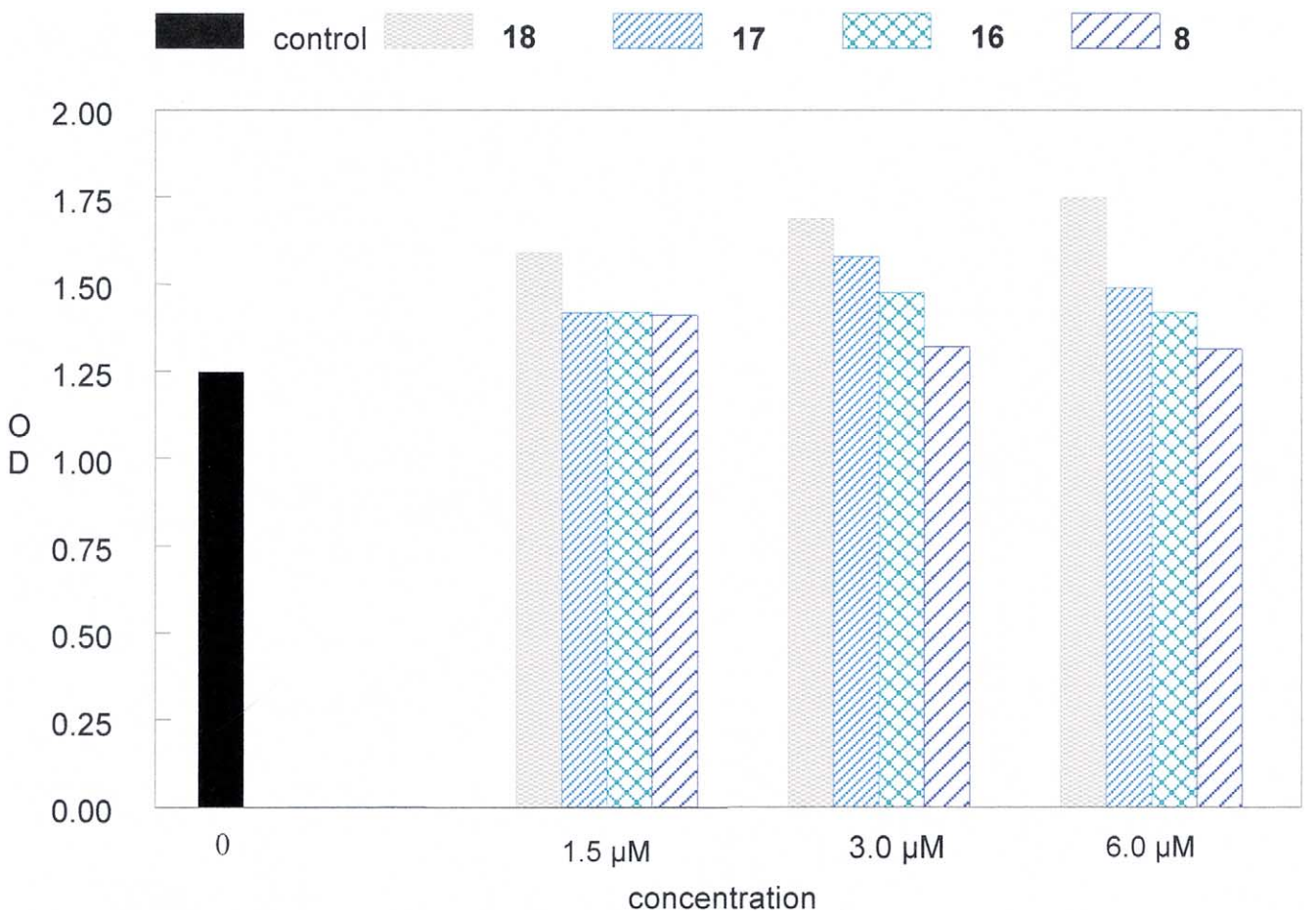


Fig. 3. Effect of PPHs in MT-4 cells.

derivatives showed higher antiviral activity. However, even if it was impossible to find a quantitative relationship between EC_{50} and log P in the case of the non-rigid analogues, the two series of derivatives did not have difference in lipophilicity so marked to justify the lack of selectivity of derivatives **23**–**41** with respect to the analogues PPHs. Moreover, all the other physical–chemical descriptors studied (molecular volume, ASA) are comparable for the two series of derivatives.

In spite of the fact that the PPHs cannot assume a butterfly-like shape, unlike the corresponding non-rigid analogues, derivatives **8**–**22** constitute a class of biologically interesting compounds. Among them for the appearance of the antiviral activity, it seems of relevance the presence of the ester function in position 2 together with that of the amino group, in position 6 or 8, and of the methoxy substituent in position 10 or 11 of the phenanthridine moiety (cfr derivatives **9**, **10**, **11**), since of the all four possible isomers three showed selectivity; whereas the presence of the acetyl group in the 2 position seems to favour the stimulating activity for lymphocyte cells (cfr compounds **16**, **17**, **18**). The exchange of the amino and methoxy groups between the two aryl moieties of the phenanthridine ring (cfr derivatives **10**, **11**, **14** and **15**), or the introduction in position 6 of an electron withdrawing group, such as azido or chlorine, (cfr derivatives **21** and **22**) resulted in a complete loss of any biological activity.

3. Conclusions

In conclusion, although only few pyrrolo[1,2-*f*]phenanthridines showed a weak selectivity against HIV-1, it was possible to evidence that also planar molecules of appropriate size can inhibit the virus, and probably interact with the binding site of NNRTIs. Moreover, the PPHs, in particular compound **10**, showed unique properties being able not only to reduce the virus-induced cytopathogenicity, but also to stimulate the growth of the same MT cells at lower concentrations. Considering that the stimulation of lymphocyte cells can be a crucial factor in *anti*-AIDS therapy, this class of compounds can be considered for further development since in the same derivative it is possible to combine stimulation of cells growth and *anti*-HIV activity.

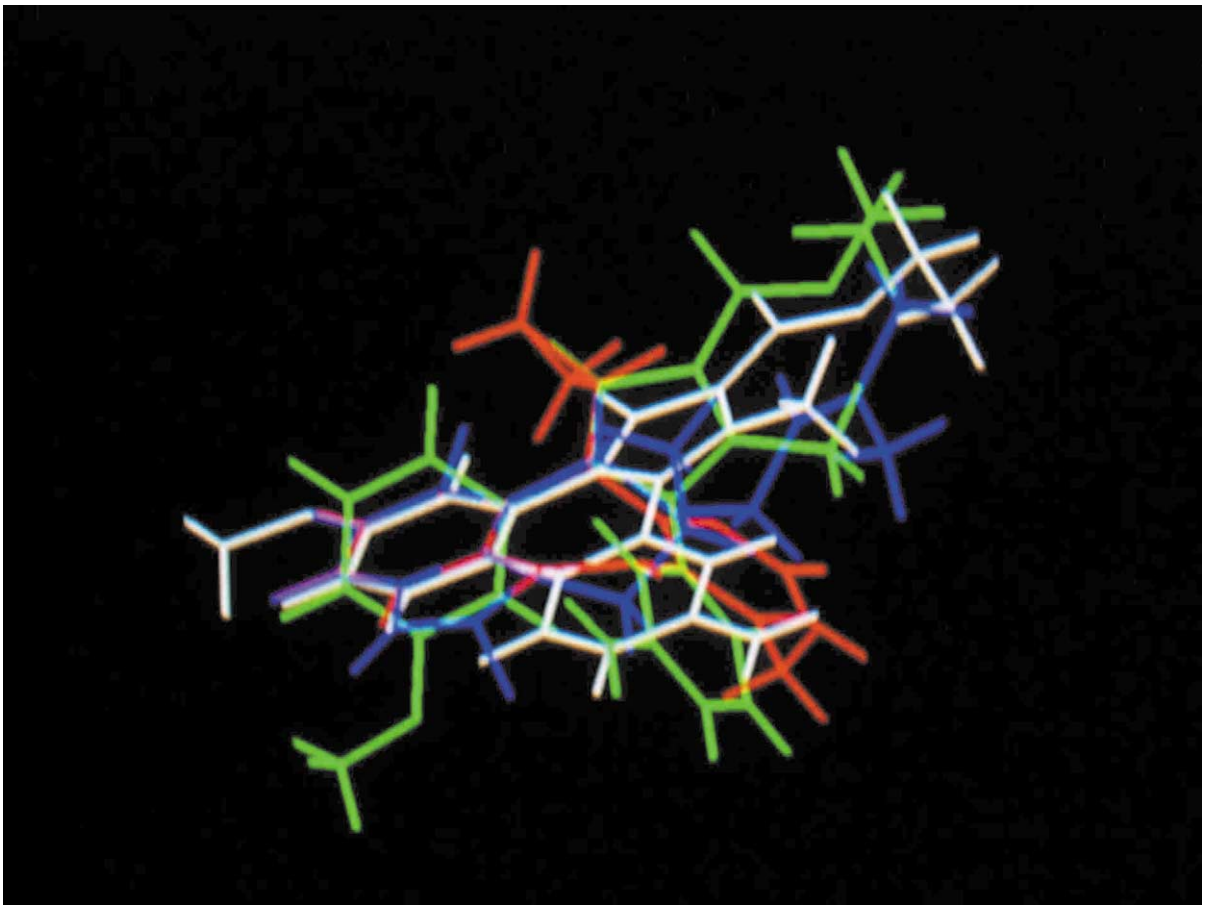


Fig. 4. Fitting between nevirapine (red), TIBO R86183 (blue), pyrrolophenanthridine (**10**) (white), and non-rigid analogue **23** (green).

4. Experimental

4.1. Chemistry

All m.p.'s were taken on a Buchi–Tottoli capillary apparatus and are uncorrected; IR spectra were determined in bromoform with a JASCO FT/IR 5300 spectrophotometer; ^{1}H- and ^{13}C-NMR spectra were measured at 200 and 50.3 MHz, respectively in $CDCl_3$ solution, unless otherwise specified, using a Bruker AC-E series 200 MHz spectrometer (TMS as internal reference); mass spectra were obtained with a HP 5890 Series II and HP 5989A-GC/MS apparatus. Column chromatography was performed with Merck silica gel 230–400 Mesh ASTM. For all new compounds analyses indicated by the symbols of the elements or functions were within $\pm 0.4\%$ of theoretical values.

2-Substituted 1-bromo-3-methylpyrrolo[1,2-f]phenanthridines **8** *and* **16** were prepared by diazotisation of 4-substituted 2-(2-aminophenyl)-3-bromo-5-methyl-1-phenylpyrroles, followed by treatment with hypophosphorous acid [1].

Substituted 3-methylpyrrolo[1,2-f]phenanthridines **9**–**13** *and* **17**–**20** were obtained by decomposition reaction of 1-(3-azidophenyl)-2-arylpyrroles **32**, **33**, **39**, **41** brought about with an excess of TFMSA [4].

Substituted ethyl 3-methylpyrrolo[1,2-f]phenanthridine-2-carboxylate **14** *and* **15** were prepared by decomposition reaction of the 2-(3-azidophenyl)-1-(3-methoxyphenyl)-pyrrole (**30**) upon treatment with TFMSA [3].

Substituted 2-(3-nitrophenyl)-1-arylpyrroles **26**, **29** *and* **36** were synthesised by reaction of 3-substituted 1-(3-nitrophenacyl)-1,4-pentandiones and suitable anilines in AcOH [3].

Substituted 2-(3-aminophenyl)-1-arylpyrroles **24** *and* **27** were obtained upon Palladium catalysed reduction of the corresponding nitro derivatives **26** and **29** [3].

Substituted 2-(3-azidophenyl)-1-arylpyrroles **28**, **30** *and* **37** were prepared upon diazotisation of the corresponding amines followed by the addition of sodium azide [3].

Substituted 1-(3-aminophenyl)-2-arylpyrroles **23**, **31**, **38**, *and* **40** were synthesised by condensation of 1-(3-substituted phenacyl)-1,4-pentandiones and 1,3-phenylendiamine in dry EtOH in the presence of *p*-toluensulfonic acid [4].

Table 3
Molecular descriptors and cytotoxicity for reference NNRTIs, PPHs, and non-rigid analogues.

Compounds	Molecular volume (Å^3)	A.S.A. (Å^2)	R_M	Log P	CC_{50} [a]
1	266.81	286.54	nd	3.0335	nd
2	233.64	273.70	nd	3.6237	nd
3	164.63	207.01	nd	1.5516	nd
4	192.17	235.29	nd	1.0310	nd
5	241.95	307.19	nd	2.8174	nd
6	195.73	240.35	nd	2.3372	nd
7	244.37	301.25	nd	3.8037	nd
8	229.24	311.76	0.513	5.1792	>300
9	216.01	305.29	0.209	3.6042	21
10	236.55	336.14	0.192	3.3515	>300
11	243.20	334.84	0.278	3.3515	>200
12	241.58	334.41	0.248	3.3515	>200
13	241.32	337.39	0.235	3.3515	>200
14	181.47	255.51	0.185	3.3515	>200
15	240.52	318.71	0.250	3.3515	>200
16	209.52	275.52	nd	4.4151	182
17	197.73	270.81	0.052	2.8401	18
18	216.72	308.42	0.087	2.5874	43
19	224.81	307.05	0.089	2.5874	40
20	222.50	302.99	0.073	2.5874	18
21	250.61	351.11	0.391	4.9806	>300
22	242.66	341.17	0.425	4.6527	>200
23	263.38	351.66	0.345	3.7137	7.2
24	263.99	348.81	0.282	3.7137	32
25	275.33	356.46	0.335	5.2887	143
26	256.17	323.87	0.507	4.7032	212
27	243.42	324.00	0.271	3.9664	>300
28	256.41	332.42	0.578	5.5955	>300
29	276.99	357.03	0.523	4.4505	33
30	277.32	368.79	0.610	5.3428	47
31	246.55	324.05	0.329	3.9664	>300
32	260.99	339.42	0.610	5.5955	176
33	278.55	365.92	0.540	5.3428	6.4
34	274.72	362.90	0.335	5.2887	10
35	272.10	345.17	0.327	5.0149	100
36	235.75	302.05	0.327	3.9391	>300
37	236.24	301.02	0.393	4.8314	18
38	222.12	290.42	0.156	3.2023	22
39	236.58	304.35	0.408	4.8314	6.3
40	242.32	328.40	0.468	2.9496	10.2
41	258.73	335.68	0.389	4.5787	8.7

nd, not determined.
[a] Compound concentration (μM) required to reduce the multiplication of MT-4 cells by 50%.

Substituted 1-(3-azidophenyl)-2-arylpyrroles **32**, **33**, **39**, *and* **41** were prepared upon diazotisation of the corresponding amines followed by the addition of sodium azide [4].

*4.1.1. Ethyl 6-azido-11-methoxy-3-methylpyrrolo[1,2-f]-phenanthridine-2-carboxylate (**21**)*

Hydrochloric acid (6 M, 2.7 mmol) was added to a suspension of the amine **10** (0.87 mmol) in water (8 mL) and the mixture was diazotised with sodium nitrite (0.87 mmol) in water (3 mL) with strict control of temperature (0–5 °C). After 30 min sodium azide (2.61 mmol) was added in small portions and the mixture was stirred for further 6 h at room temperature (r.t.). The solid precipitate was filtered off, air dried and purified by column chromatography, eluant dichloromethane (DCM), to give **21** (yield 90%), m.p. 160 °C, IR: 2106 (N_3), 1697 (CO) cm^{-1}; ^{1}H-NMR: δ 1.44 (3H, t, $J = 7.1$ Hz, CH_3), 3.15 (3H, s, CH_3), 3.94 (3H, s, CH_3), 4.38 (2H, q, $J = 7.1$ Hz, CH_2), 6.96 (1H, dd, $J = 8.9$, 2.6 Hz, H-10), 7.03 (1H, dd, $J = 8.7$, 2.2 Hz, H-7), 7.26 (1H, s, H-1), 7.30 (1H, d, $J = 2.6$ Hz, H-12), 7.85 (1H, d, $J = 2.2$ Hz, H-5), 7.94 (1H, d, $J = 8.9$ Hz, H-9), 8.11 (1H, d, $J = 8.7$ Hz, H-8); ^{13}C-NMR: δ 14.56 (q), 16.52 (q), 55.46 (q), 60.03 (t), 103.34 (d), 104.47 (d), 108.38 (d), 115.14 (d), 115.41 (d), 116.66

(s), 118.04 (s), 121.11 (s), 123.73 (d), 124.75 (d), 127.36 (s), 129.09 (s), 133.14 (s), 134.72 (s), 138.33 (s), 159.68 (s), 165.47 (s). Anal. ($C_{21}H_{18}N_4O_3$) C, H, N.

4.1.2. Ethyl 6-chloro-11-methoxy-3-methylpyrrolo[1,2-f]phenanthridine-2-carboxylate (22)

To a solution of *iso*-amyl nitrite (1.43 mmol) in dry MeCN (5 mL), anhydrous copper(II) chloride (1.7 mmol) was added at 0 °C. A suspension of the amine **10** (1.43 mmol) in dry MeCN (15 mL) was slowly added over a period of 5 min, at 0–5 °C, to the resulting solution. The reactants were stirred at r.t. until disappearence of the starting material (tlc monitorage, 2 h). The organic layer was evaporated under reduced pressure to give a residue, which was purified by column chromatography using light petroleum ether (b.p. 50–70 °C)–EtOAc 9:1 as eluant. From the complex reaction mixture it was possible to isolate only compound **22** (yield 31%) which was recrystallised from EtOH, m.p. 165 °C, IR: 1703 (CO) cm^{-1}; ^{1}H-NMR: δ 1.44 (3H, t, $J = 7.8$ Hz, CH_3), 3.03 (3H, s, CH_3), 3.84 (3H, s, CH_3), 4.43 (2H, q, $J = 7.8$ Hz, CH_2), 6.84 (1H, dd, $J = 8.9$, 2.9 Hz, H-10), 7.07 (1H, s, H-1), 7.09 (1H, d, $J = 2.9$ Hz, H-12), 7.18 (1H, d, $J = 8.8$ Hz, H-7), 7.75 (1H, d, $J = 8.9$ Hz, H-9), 7.87 (1H, d, $J = 8.8$ Hz, H-8), 8.01 (1H, s, H-5); ^{13}C-NMR: δ 14.50 (q), 16.34 (q), 55.31 (q), 59.96 (t), 103.16 (d), 104.08 (d), 115.22 (d), 116.35 (s), 117.53 (d), 122.29 (s), 123.64 (d), 124.14 (d), 124.36 (d), 126.93 (s), 128.68 (s), 131.75 (s), 133.04 (s), 152.38 (s), 158.66 (s), 159.65 (s), 165.36 (s); ms: m/z 367/369. Anal. ($C_{21}H_{18}ClNO_3$) C, H, Cl, N.

4.1.3. Ethyl 5-(3-bromophenyl)-1-(3-methoxyphenyl)-2-methylpyrrole-3-carboxylate (25)

This compound was prepared from amine **24** upon treatment with *iso*-amyl nitrite and anhydrous copper(II) bromide according to the procedure described for derivative **22**. It was purified by column chromatography using DCM as eluant, (yield 39%), IR: 1694 cm^{-1} (CO); ^{1}H-NMR (DMSO-d_6): δ 1.29 (3H, t, $J =$ 7.1 Hz, CH_3), 2.33 (3H, s, CH_3), 3.74 (3H, s, CH_3), 4.23 (2H, q, $J = 7.1$ Hz, CH_2), 6.58–6.79 (2H, m, ArH), 6.82 (1H, s, H-4), 6.87–7.36 (6H, m, ArH); ^{13}C-NMR (DMSO-d_6): δ 12.15 (q), 14.44 (q), 55.50 (q), 59.10 (t), 110.36 (d), 112.94 (s), 114.27 (d), 114.50 (d), 120.25 (s), 120.55 (d), 121.42 (s), 126.37 (d), 129.30 (d), 129.92 (d), 130.20 (d), 130.32 (d), 131.56 (s), 134.09 (s), 138.23 (s), 159.84 (s), 164.26 (s); ms: m/z 413/415. Anal. ($C_{21}H_{20}BrNO_3$) C, H, Br, N.

4.1.4. Ethyl 5-(3-methoxyphenyl)-2-methyl-1-(3-X-phenyl)pyrroles-3-carboxylate (34, 35)

A solution of ethyl 1-(3-methoxyphenacyl)-1,4-pentandione-3-carboxylate (60 mmol) [16], the suitable 3-haloaniline (60 mmol) and *p*-toluensulfonic acid (0.26 mmol) in dry EtOH (60 mL) was heated under reflux for 2 h. After cooling, the resultant brown solution was evaporated under reduced pressure and the residue chromatographed using light petroleum ether (b.p. 50–70 °C)–EtOAc 8:2 as eluant.

34 (X = Br) (yield 49%), IR: 1694 (CO) cm^{-1}; ^{1}H-NMR: δ 1.38 (3H, t, $J = 7.3$ Hz, CH_3), 2.41 (3H, s, CH_3), 3.64 (3H, s, CH_3), 4.32 (2H, q, $J = 7.3$ Hz, CH_2), 6.55–6.73 (3H, m, ArH), 6.79 (1H, s, H-4), 7.22–7.54 (5H, m, ArH); ^{13}C-NMR: δ 12.46 (q), 14.50 (q), 54.99 (q), 59.59 (t), 110.35 (d), 112.89 (d), 113.17 (d), 120.56 (d), 122.47 (s), 123.18 (s), 127.32 (d), 129.14 (d), 130.41 (d), 131.48 (d), 131.50 (d), 133.13 (s), 133.70 (s), 137.90 (s), 139.44 (s), 159.20 (s), 177.34 (s); ms: m/z 413/415. Anal. ($C_{21}H_{20}BrNO_3$) C, H, Br, N.

35 (X = Cl) (yield 60%), IR: 1694 (CO) cm^{-1}; ^{1}H-NMR: δ 1.37 (3H, t, $J = 6.8$ Hz, CH_3), 2.41 (3H, s, CH_3), 3.74 (3H, s, CH_3), 4.32 (2H, q, $J = 6.8$ Hz, CH_2), 6.55–6.73 (3H, m, ArH), 6.79 (1H, s, H-4), 7.22–7.54 (5H, m, ArH); ^{13}C-NMR: δ 12.41 (q), 12.51 (q), 55.43 (q), 59.55 (t), 110.69 (d), 113.00 (s), 114.13 (d), 114.23 (d), 120.69 (d), 125.74 (d), 126.44 (d), 127.78 (d), 129.17 (d), 129.79 (s), 129.99 (d), 132.23 (s), 133.87 (s), 134.08 (s), 138.67 (s), 160.16 (s), 165.32 (s); ms: m/z 369/371. Anal. ($C_{21}H_{20}ClNO_3$) C, H, Cl, N.

4.2. Biological assays

4.2.1. Compounds

Test compounds were dissolved in DMSO at an initial concentration of 200 mM and then were serially diluted in culture medium.

4.2.2. Cells

Cell lines were from American Type Culture Collection (ATCC). H9/IIIB and MT-4 cells [grown in RPMI 1640 containing 10% foetal calf serum (FCS), 100 UI mL^{-1} penicillin G and 100 μg mL^{-1} streptomycin] were used for *anti*-HIV-1 assays. Cell cultures were checked periodically for the absence of mycoplasma contamination with a MycoTect Kit (Gibco).

4.2.3. Viruses

Human immunodeficiency virus type-1 (HIV-1, III_B strain) was obtained from supernatants of persistently infected H9/III_B cells. HIV-1 stock solutions had a titre of 5×10^7 cell culture infectious dose 50 ($CCID_{50}$) mL^{-1}.

4.2.4. Antiviral assays

Activity against the HIV-1 multiplication in acutely infected cells was based on inhibition of virus-induced cytopathogenicity in MT-4 cells [17]. Briefly, 50 μL of RPMI 10% FCS containing 1×10^4 cells were added to each well of flat-bottomed microtiter trays containing 50 μL of medium and serial dilutions of test compounds. Twenty microlitre of an HIV-1 suspension containing 100 $CCID_{50}$ were then added. After a 4-day

incubation at 37 °C, the number of viable cells was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) method [18,19]. Cytotoxicity of compounds, based on the viability of mock-infected cells as monitored by the MTT and methylene blue methods [14], was evaluated in parallel with their antiviral activity.

4.2.5. Estimation of cell growth

Culture supernatants were aspirated and cells fixed and stained by adding a solution of methylene blue (5 g L^{-1} of 50% (v/v) ethanol–water, 0.1 mL per well) to each well. After 30 min at r.t., plates were inverted briefly to allow most of the stain to drain away. Unbound stain was washed off by immersing trays in water (3–4 sequential rinses). Plates were air-dried and stored until required for further processing. Stained cells were solubilised overnight using 1% Sarkosyl (Sigma) in phosphate-buffered saline (0.1 mL per well). Absorbances were read by an ELISA plate spectrophotometer at a sample wavelength of 620 nm and a reference wavelength of either 405 nm ('Titertek Multiskan' spectrophotometer, Flow), or 486 nm (SLT 210 spectrophotometer, Salzburger Electronik Industrie). The CC_{50} is defined as that concentration of drug, which decreases absorbance to 50% of that in control (drug-free) cultures.

The cultures were incubated in growth medium, in the absence or in the presence of various dilutions of test compounds. After a 4-day incubation at 37 °C, the cell number was determined with a Coulter counter and was corrected for viability, as determined by trypan blue exclusion [15].

4.2.6. Linear regression analysis

Viral and cell growth at each drug concentration was expressed as percentage of untreated controls and the concentrations resulting in 50% (EC_{50}, CC_{50}) growth inhibition was determined by linear regression analysis.

4.3. Lipophilicity measurements

The relative lipophilicity of the compounds was measured by reversed-phase thin-layer chromatography according to the method previously described [20]. R_M values were calculated from the experimental R_f values (calculated as mean values for five determinations) according to the equation $R_M = \log[(1/R_f) - 1]$. Higher R_M values correspond to higher lipophilicity. Predicted log P values were calculated with the software TSAR V2.22 [13], using the atomic log P values determined according to Ref. [21].

Acknowledgements

This work was financially supported in part by Ministero dell'Università e della Ricerca Scientifica, by Consiglio Nazionale delle Ricerche and by grants from Regione Autonoma Sardegna (Progetto Biotecnologie).

References

[1] G. Dattolo, G. Cirrincione, A.M. Almerico, E. Aiello, I. D'Asdia, J. Heterocycl. Chem. 23 (1986) 1371–1373.
[2] G. Cirrincione, G. Dattolo, A.M. Almerico, G. Presti, E. Aiello, Heterocycles 24 (1986) 3403–3410.
[3] A.M. Almerico, G. Cirrincione, G. Dattolo, E. Aiello, F. Mingoia, J. Heterocycl. Chem. 31 (1994) 193–198.
[4] A.M. Almerico, G. Cirrincione, P. Diana, S. Grimaudo, G. Dattolo, E. Aiello, F. Mingoia, P. Barraja, Heterocycles 37 (1994) 1549–1559.
[5] E. Aiello, G. Dattolo, G. Cirrincione, A.M. Almerico, P. Diana, S. Grimaudo, F. Mingoia, P. Barraja, Il Farmaco 50 (1995) 365–368.
[6] G.V. De Lucca, M.J. Otto, Bioorg. Med. Chem. Lett. 2 (1992) 1639–1644.
[7] G. Campiani, V. Nacci, I. Fiorini, M.P. De Filippis, A. Garofalo, G. Greco, E. Novellino, S. Altamura, L. Di Renzo, J. Med. Chem. 39 (1996) 2672–2680.
[8] M. Artico, G. Stefancich, R. Silvestri, S. Massa, E. Pagnozzi, A.G. Loi, D. Musu, M. Doa, P. Scano, P. La Colla, Med. Chem. Res. 4 (1994) 283–290.
[9] T. Antonucci, J.S. Warmus, J.C. Hodges, D.G. Nickell, Antiviral Chem. Chemother. 6 (1995) 98–108.
[10] J. Ding, K. Das, H. Moereels, L. Koymans, K. Andries, P.A.J. Janssen, S.H. Hughes, E. Arnold, Nature Struct. Biol. 2 (1995) 407–415.
[11] C. Mao, R. Vig, T.K. Venkatachalam, E.A. Sudbeck, F.M. Uckun, Bioorg. Med. Chem. Lett. 8 (1998) 2213–2218.
[12] M.P. Doyle, B. Siegfried, J.F. Dellaria Jr., J. Org. Chem. 42 (1977) 2426–2430.
[13] The software packages were supplied by Oxford Molecular; the calculations were run on a Silicon Graphics work station Indigo2 R4400.
[14] G.J. Finlay, B.C. Baguley, W.R. Wilson, Anal. Biochem. 139 (1984) 272–277.
[15] A. Pani, M.E. Marongiu, P. La Colla, Antiviral Res. 22 (1993) 31–43.
[16] G. Dattolo, G. Cirrincione, A.M. Almerico, G. Presti, E. Aiello, Heterocycles 22 (1984) 2269–2276.
[17] A. Mai, M. Artico, G. Sbardella, S. Quartarone, S. Massa, A.G. Loi, A. De Montis, F. Scintu, M. Putzolu, P. La Colla, J. Med. Chem. 40 (1997) 1447–1454.
[18] R. Pauwels, J. Balzarini, M. Baba, R. Snoeck, D. Schols, P. Herdewijn, J. Desmyter, E. De Clerq, J. Virol. Methods 20 (1988) 309–321.
[19] F. Denizot, R. Lang, J. Immunol. Methods 89 (1986) 271–277.
[20] G. Cirrincione, A.M. Almerico, G. Dattolo, E. Aiello, S. Grimaudo, P. Diana, Il Farmaco 47 (1992) 1555–1562.
[21] V.N. Viswanadhan, A.K. Ghose, G.R. Revankar, R.K. Robin, J. Chem. Inf. Comput. Sci. 29 (1989) 163–172.