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Indolo[3,2-*c*]cinnolines with Antiproliferative, Antifungal, and Antibacterial Activity

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Abstract—A series of indolo[3,2-*c*]cinnoline derivatives was prepared and tested to evaluate their biological activity. Most of them inhibited the proliferation of leukemia, lymphoma and solid tumor-derived cell lines at micromolar concentrations, whereas none of the compounds were active against HIV-1. With the exception of **7g**, all title compounds showed antibacterial activity against gram-positive bacteria, being up to 200 times more potent than the reference drug streptomycin. Some of the indolo[3,2-*c*]cinnolines were also endowed with good antifungal activity, particularly against *Cryptococcus neoformans*.

Introduction

Polycyclic nitrogen heterocycles can be good pharmacophores for classes of antineoplastic drugs because of their potential ability to bind to DNA by intercalating between the base pairs of the DNA duplex. Acridine and phenanthridine derivatives of type **1** and **2** (Fig. 1) are well known intercalating agents.[1] In particular, in the series of 9-acrididinylmethanesulfonanilide derivatives **1** ($R = NHSO_2Me$) and of ethidium derivatives **2** ($R = Et$), it has been demonstrated that the biological activity is related to the presence of certain substituents at positions suitable for interaction with DNA base pairs.[2] The anthracycline class of antitumor antibiotics **3** also tightly binds DNA by intercalation and shows strong specificity for GC, with interaction proposed between the drugs and the 2-amino group of guanine.[3]

We have recently described the synthesis and biological activity of the new ring system indolo[1,2-*c*]benzo-[1,2,3]triazine **4**.[4] All indolo-benzotriazines **4a–g** have been evaluated for antiproliferative activity against a panel of leukemia-, lymphoma-, carcinoma- and neuroblastoma-derived cell lines. These compounds proved inhibitory to the proliferation of T and B cell lines at micromolar concentrations (IC_{50} range 0.08–13 μM), whereas their potency of antiproliferative activity against solid tumor cell lines was rather low. When evaluated for antifungal activity, **4a**,**d** were inhibitory to some of the fungi tested, although at concentrations comparable to those cytotoxic for human cells. All compounds were highly inhibitory to *Streptococcus* and *Staphylococcus*, and **4b**,**c**,**g** resulted up to 80 times more potent than the reference drug streptomycin.

Closely related to the above compounds is the class of the indolo[3,2-*c*]cinnoline of type **7**. We have recently reported a new synthesis of this ring system and have presented some evidence for their in vitro antileukemic activity.[5] Now we describe the synthesis of additional derivatives bearing suitable substituents in the opportune position, that can help in the interaction with DNA, and the interesting results of a wider biological screening.

Chemistry

Our approach to the indolo[3,2-*c*]cinnoline nucleus involved the construction of the cinnoline ring on the

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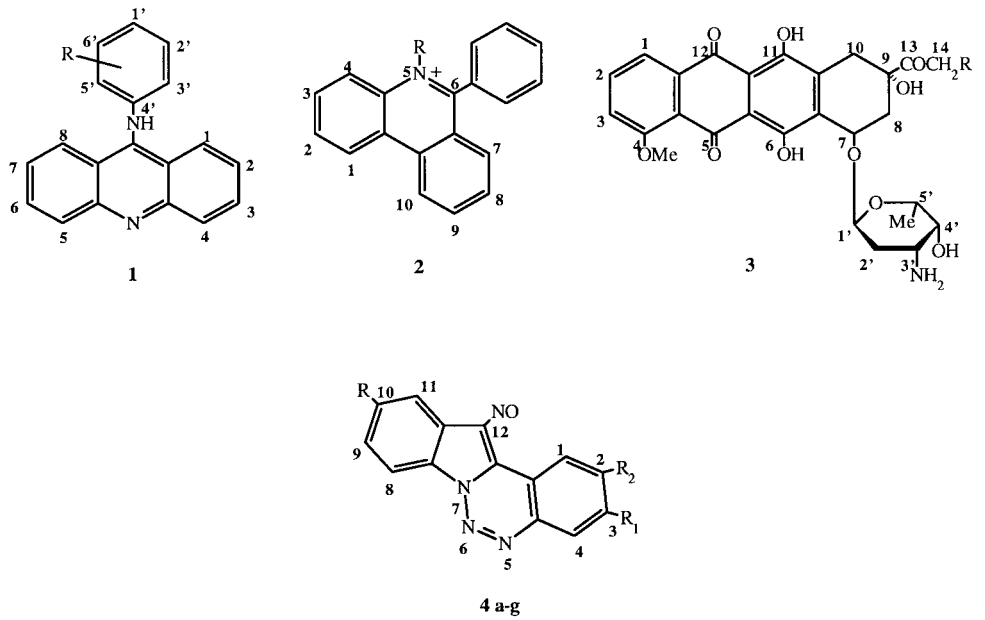
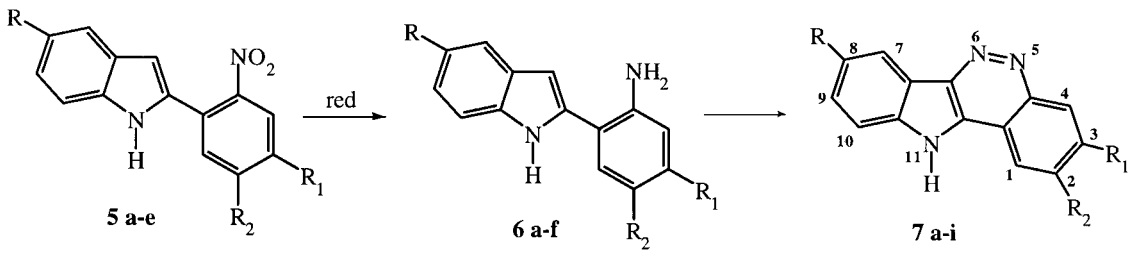


Figure 1. (a) R=R_1=R_2=H; (b) R=R_1=H, R_2=Cl; (c) R=OMe, R_1=R_2=H; (d) R=R_2=H, R_1=Cl; (e) R=R_1=H, R_2=Me; (f) R=Cl, R_1=R_2=H; (g) R=NO_2, R_1=R_2=H.

indole moiety by using as key intermediates suitably substituted 2-aminophenylindoles of type **6**. This synthetic route allows easy functionalization of either the indole ring and/or the cinnoline moiety with substituents required for DNA-binding. We have recently explored and compared the synthetic routes leading to these compounds in preparative scale on the basis of considerations about (a) low costing and easily available starting materials, (b) easy manipulation of the reaction mixture, (c) formation of only one indole derivative, (d) high yields.[4,5] The most advantageous approach involved the easy preparation of 2-(2-nitroaryl)-indoles **5a–e** by an intramolecular Wittig reaction of ylides, accessible in turn, in good yields (71–91%), from 2-aminobenzyl-triphenylphosphonium salts and 2-nitrobenzoyl chlorides. Only in the case of the preparation of derivative **6f** the classical Fisher reaction, starting from 2-aminoacetophenone and 4-chlorophenylhydrazine, was preferred because of higher yield. In all the other cases, reduction of the nitro group, with iron in acetic acid or catalytically with Pd and hydrogen, gave good yields of the amino derivatives **6a–e** (Scheme 1). The diazotization reaction of the amines **6a–f**, carried out at 0°C in acetic acid with stoichiometric amount of sodium nitrite, immediately followed by the coupling on the 3 position of the indole moiety gave, in high yields, the corresponding indolo[3,2-*c*]cinnoline derivatives **7a–f**. The 8-nitroindolo[3,2-*c*]cinnolines **7h,i** were obtained from derivative **7a**,**b** upon treatment with potassium nitrate in sulfuric acid, whereas halogenation of compound **7a** with NBS in DMF afforded the 8-bromoindolo[3,2-*c*]cinnoline **7g**, in good yield.

Results and Discussion

The antiproliferative activity of indolo-cinnolines **7a–i** was evaluated against a panel of cell lines derived from human tumors such as leukemias, lymphomas, carcinomas and neuroblastomas (Table 1). Although less potently than doxorubicin (Doxo, **3**, R=OH), the title compounds inhibited the proliferation of T and B cell lines at micromolar concentrations. The most potent derivative was **7h**, which showed antiproliferative activity in the submicromolar range (IC_{50} range 0.08–1.2 μM), followed, in decreasing order of potency, by **7b** (IC_{50} range 0.4–1.0 μM), **7i** (IC_{50} range 0.2–2.2 μM), **7c** (IC_{50} range 1.0–17 μM), **7a** (IC_{50} range 2.2–12 μM), and **7e** (IC_{50} range 2.3–50 μM). Compounds **7d** and **7f** showed a moderate activity (IC_{50} range 5–50 μM),



Scheme 1. (a) R=R_1=R_2=H; (b) R=R_2=H, R_1=Cl; (c) R=R_1=H, R_2=Cl; (d) R=R_1=H, R_2=Me; (e) R=OMe, R_1=R_2=H (f) R=Cl, R_1=R_2=H; (g) R=Br, R_1=R_2=H; (h) R=NO_2, R_1=R_2=H; (i) R=NO_2, R_1=Cl, R_2=H.

Table 1. Antiproliferative activity of indolo[3,2-*c*]cinnolines

Cell lines	IC_{50} [μM][a]									
	7a	**7b**	**7c**	**7d**	**7e**	**7f**	**7g**	**7h**	**7i**	Doxo
Leukemia/lymphoma										
L1210	12	1.0	3.5	30	50	24	> 100	0.3	ND	0.23
Wil2-NS	4.5	0.5	1.0	20	10	18	89	0.2	1.1	0.02
CCRF-SB	5	0.7	2.8	21	3.2	27	> 100	0.2	1.1	0.01
Raji	6.8	0.8	17	> 10	12	> 10	> 100	1.2	1.8	0.08
CCRF-CEM	5	0.5	1.9	10	7.3	5.0	54	0.08	2.2	0.10
MOLT-4	3.3	0.4	1.6	12	3.2	12	> 100	0.08	0.2	0.03
MT-4	2.2	0.9	4.7	25	2.3	50	> 100	0.3	0.5	0.09
Carcinoma										
HT-29	50	5.2	0.8	2.8	> 50	2.1	94	1.9	4.5	0.05
HeLa	8.9	1.0	10	48.1	33	> 10	> 100	7.7	1.0	0.25
ACHN	> 5.0	3.1	1.2	41.5	15.5	> 10	> 100	3.0	3.0	0.44
5637	4.0	0.3	2.1	10	19	> 10	18.9	0.7	2.7	0.02
Neuroblastoma										
IMR-32	5.8	0.6	ND	ND	7.0	ND	ND	ND	ND	< 0.01

[a] Compound concentration required to reduce cell multiplication by 50% under conditions allowing untreated controls to undergo at least three consecutive rounds of multiplication. L1210, mouse leukemia; Wil2-NS, human splenic B-lymphoblastoid cells; CCRF-SB, human acute B-lymphoblastoic leukemia; Raij, human Burkitt lymphoma; CCRF-CEM and MOLT-4, human acute T-lymphobastic leukemia; MT-4, human $CD4^+$ T-cells containing an integrated HTLV-1 genome; HT-29, human colon adenocarcinoma; HeLa, human cervix carcinoma; ACHN, human renal adenocarcinoma; 5637, human bladder carcinoma; IMR-32, human neuroblastoma. ND: not determined.

whereas **7g** was substantially inactive (IC_{50} range 54–100 μM). The antiproliferative activity of the test compounds against solid tumor cell lines was lower than against B and T cell tumors. In this case the most active compounds were **7b**, **7h** and **7i**, which showed an IC_{50} ranges comparable to that of *cis*-platinum (1–8 μM).

In order to get more insights into their cytotoxicity for normal human cells, **7b** and **7h** were assayed in vitro against peripheral blood lymphocytes (PBL) from healthy donors. Doxorubicin, MTX, *cis*-platinum and taxol were run as reference drugs (Table 2).

All compounds proved cytotoxic for both PHA-stimulated PBL and resting PBL at concentrations close to those active against leukemic cell lines. It is noteworthy that only the antimitotic drug taxol was noncytotoxic for resting PBL, whereas MTX, *cis*-platinum and doxorubicin proved equally cytotoxic for proliferating and resting lymphocytes.

When tested against the Human Immunodeficency Virus type-1 (HIV-1), no activity was found at concentrations below those cytotoxic for uninfected MT-4 cells (results not shown). Negative results were also obtained against Herpes Simplex Virus type-1, Coxsackie, and Vesicular Stomatitis Virus. Test compounds were evaluated for antifungal activity (Table 3) in comparison with miconazole, used as reference drug. Compounds **7d**, **7e**, **7f** and **7g** were totally devoid of activity up to 200 μM, whereas compound **7h** was inhibitory to all fungal species tested, with spectrum and potency comparable to those of miconazole. The other derivatives were moderately active. The most sensitive species was *Cryptococcus neoformans*, against which **7a**, **7b** and **7h** showed a potency (MIC 0.1–1.6 μM) comparable, if not higher, than that of miconazole (MIC 0.9 μM).

The antibacterial activity of indolo-cinnolines was evaluated against representatives of gram-positive and gram-negative human pathogens (Table 4). In general, test compounds were inactive against *Salmonella* and *Shigella*; the sole exception was derivative **7h**, which inhibited also the gram-negative bacteria with potency superior to that of streptomycin. With the exception of **7g**, the indolocinnolines were potent inhibitors of gram-positive bacteria (*Streptococcus* and *Staphylococcus*). The most potent derivative was **7h** (MIC 0.05–0.02 μM), followed by **7b** (MIC 0.1–0.07 (μM), **7a** (MIC 0.2–0.1 μM), **7i** (MIC 0.3–0.09 μM), and **7c** (MIC 0.4 μM), all being far more potent than the reference drug streptomycin.

In terms of SAR studies, similarly to that observed for indolo-benzotriazines, the antiproliferative effect is

Table 2. Comparative cytoxicity of indolo[3,2-*c*]cinnolines and reference anticancer drugs for normal human lymphocytes

Cell lines	CC_{50} [μM][a]					
	7b	**7h**	MTX	*cis*-Pt	Doxo	Taxol
PBL_{PHA}[b]	0.2	0.5	0.05 ± 0.005	10 ± 0.9	0.04 ± 0.06	10 ± 2.5
$PBL_{resting}$[c]	0.9	0.8	0.1 ± 0.01	16 ± 1	0.09 ± 0.01	16 ± 2.9
Leukemia[d]	0.7	0.3	0.02	2.6	0.06	8.3

[a] Compound concentration (± SD) required to reduce cell multiplication (PBL) by 50%.
[b] PBL were stimulated with PHA and then resuspended in IL_2-containing medium in the presence of drugs.
[c] PBL were treated with test drugs for 3 days, then were stimulated with PHA and resuspended in drug-free medium.
[d] Data are the mean IC_{50} values obtained with leukemia cell lines.

Table 3. Antifungal activity of indolo[3,2-*c*]cinnolines

Species	MIC [μM][a]									
	7a	**7b**	**7c**	**7d**	**7e**	**7f**	**7g**	**7h**	**7i**	Miconazole
C. albicans	150	9.4	12	> 200	> 200	> 200	> 200	1.6	66	7.5
C. parapsilosis	1.8	2.3	100	> 200	> 200	> 200	> 200	1.6	22	0.9
C. paratropicalis	37	19	50	> 200	> 200	> 200	> 200	1.6	66	7.5
C. neoformans	0.8	0.1	6.2	> 200	> 200	> 200	> 200	1.6	7.4	0.9
T. mentagrophytes	1.8	75	ND	ND	> 200	ND	ND	ND	ND	0.9
A. fumigatus	5.0	4.7	200	> 200	> 200	> 200	> 200	3.1	200	1.9

[a] Minimum inhibitory concentration. ND: not determined.

Table 4. Antibacterial activity indolo[3,2*c*]cinnolines

Species	MIC [μM][a]									
	7a	**7b**	**7c**	**7d**	**7e**	**7f**	**7g**	**7h**	**7i**	Streptomycin
Streptococcus	0.2	0.1	0.4	6.2	3.1	6.2	> 200	0.05	0.3	2.1
Staphlyococcus	0.1	0.07	0.4	6.2	6.2	6.2	100	0.02	0.09	4.2
Salmonella	> 200	> 200	> 200	> 200	> 200	> 200	> 200	3.1	200	8.6
Shigella	> 200	> 200	> 200	> 200	> 200	> 200	> 200	1.6	66	2.1

[a] Minimum inhibitory concentration.

peculiar of this class of compounds (see activity of **7a**). Also in this case, the potency can be modulated by introducing different substituents at different positions of the molecule. In particular, the R substituent seems to be critical. In fact whereas the introduction of a nitro group confers the maximum potency (**7h**), the introduction of a chlorine atom (**7f**) or a methoxy group (**7e**) leads to a decrease in the activity. The presence of a bromine atom (**7g**) resulted instead in the least active derivative. The antiproliferative activity could also be increased by a chlorine atom in R_1, as for **7b** or a chlorine atom in R_1 and a nitro group in R, as for **7i**, which resulted only slightly inferior to **7h**. Although less markedly, potency varied also according to the R_2 substituent. In this position, the introduction of a chlorine atom (**7c**) slightly increased potency with respect to the unsubstituted derivative **7a**, whereas, on the contrary, the presence of a methyl group (**7d**) reduced the antiproliferative activity.

As far as the antifungal activity is concerned, the results indicate that, also in the case of the indolo-cinnoline molecule, some positions are crucial for the activity. With the sole exception of $R=NO_2$, which gives rise to the most potent and wide spectrum derivative **7h**, all the other R substituents (Cl, Br, OMe) determine the complete loss of activity (**7f**, **7g**, **7e**, respectively). Unlike what has been observed with indolo-benzotriazines, the introduction of a chlorine atom in R_1 increases the activity with respect to the unsubstituted derivative (compare **7b** and **7a**), whereas the same halogen in R_2 reduces it (compare **7c** and **7a**). The introduction of a nitro group in R and a chlorine atom in R_1 reduces the activity (compare **7i** and **7a**). The activity is also lost when a methyl group is introduced in R_2 (**7d**).

The antibacterial activity of indolo-cinnolines, unlike what has been observed with indolo-benzotriazines, can be modulated by modification of the lead compound **7a**. Against gram-positive bacteria, the activity was strongly increased with $R=NO_2$ (**7h**), whereas it was significantly reduced with R=Cl or OMe (**7f** and **7e** respectively) and completely lost with R=Br (**7g**). The activity was superior to that of **7a** also when R_1 was a chlorine atom (**7b**), but it was decreased when the chlorine atom was introduced in R_2 (**7c**). The introduction of a nitro group in R and a chlorine atom in R_1 does not increase the activity with respect to the unsubstituted derivative (compare **7i** and **7a**). A more marked decrease in potency was observed when the chlorine in this position was replaced by a methyl group (**7d**). Finally, with respect to gram-negative bacteria, the unsubstituted indolo-cinnoline **7a** did not show activity but, interestingly, a nitro group in R conferred to the molecule antibacterial activity comparable to that of streptomycin.

Experimental

Chemistry

All melting points were taken on a Buchi–Tottoli capillary apparatus and are uncorrected; IR spectra were determined in bromoform with a Jasco FT/IR 5300 spectrophotometer; ^1H and ^{13}C NMR spectra were measured at 200 and 50.3 MHz respectively in $(CD_3)_2SO$ solution using a Bruker AC-E series 200 MHz spectrometer (TMS as internal reference). Column chromatography was performed with Merck silica gel 230–400 Mesh ASTM. Satisfactory microanalyses were obtained for all the new compounds (C ± 0.30, H ± 0.10, N ± 0.14).

Preparation of substituted indolo[3,2-*c*]cinnolines 7a–f. To a suspension of the amines **6a–f**[4,5] (5 mmol) in acetic acid (30 mL) sodium nitrite (5 mmol) in water (2 mL) was added dropwise at 0–5°C with stirring. The mixture was stirred for 4 additional h at room temperature and

then neutralized with an aqueous solution of sodium hydroxide (5%). The solid precipitate was filtered off, air dried and recrystallized from ethanol. Substituted indolo[3,2-*c*]cinnolines **7a**–**c,e** have already been described by us.[5] 2-Methylindolo[3,2-*c*]cinnoline (**7d**) had mp >320°C (80% yield), IR: 3425 (broad NH) cm^{-1}; ^1H NMR ppm: 2.70 (s, 3H, CH_3), 7.47 (dt, $J=7.4$, 1.2 Hz, 1H, H-8), 7.64 (dt, $J=7.4$, 1.2 Hz, 1H, H-9), 7.75 (dd, $J=7.4$, 1.2 Hz, 1H, H-10), 7.78 (d, $J=8.5$ Hz, 1H, H-4), 8.31 (d, $J=2.0$ Hz, 1H, H-1), 8.46 (dd, $J=8.5$, 2.0 Hz, 1H, H-3), 8.50 (dd, $J=7.4$, 1.2 Hz, 1H, H-7), 12.93 (s, 1H, NH). Anal. calcd for $C_{15}H_{11}N_3$: C, 77.23; H, 4.75; N, 18.02. Found: C, 77.35; H, 4.80; N, 17.98. 8-Chloroindolo[3,2-*c*]cinnoline (**7f**) had mp >320°C (80% yield), IR: 3429 (broad NH) cm^{-1}; ^1H NMR ppm: 7.65 (dd, $J=8.8$, 2.0 Hz, 1H, H-9), 7.82 (d, $J=8.8$ Hz, 1H, H-10), 7.94 (dd, $J=6.9$, 1.5 Hz, 1H, H-1), 7.98 (dd, $J=6.9$, 1.5 Hz, 1H, H-4), 8.51 (dt, $J=6.9$, 1.5 Hz, 1H, H-3), 8.57 (d, $J=2.0$ Hz, 1H, H-7), 8.59 (dt, $J=6.9$, 1.5 Hz, 1H, H-2), 13.85 (bs, 1H, NH). Anal. calcd for $C_{14}H_8N_3Cl$: C, 66.28; H, 3.18; N, 16.56. Found: C, 66.35; H, 3.20; N, 16.48.

Preparation of 8-bromoindolo[3,2-*c*]cinnoline (7g). To a suspension of the indolo[3,2-*c*]cinnoline (**7a**) (2.3 mmol) in DMF (30 mL), a solution of NBS (2.3 mmol) in DMF (8 mL) was added dropwise at room temperature. After stirring for 2 h, the reaction mixture was poured onto crushed ice. The solid was filtered off, air dried and recrystallized from ethanol to give **7g**, mp >320°C (64% yield), IR: 3441 (broad NH) cm^{-1}; ^1H NMR ppm: 7.75 (dd, $J=8.6$, 1.8 Hz, 1H, H-9), 7.85 (d, $J=8.6$ Hz, 1H, H-10), 7.93 (dd, $J=6.7$, 1.5 Hz, 1H, H-1) 7.98 (dd, $J=6.7$, 1.5 Hz, 1H, H-4), 8.50 (dt, $J=6.7$, 1.5 Hz, 1H, H-3), 8.61 (d, $J=1.8$ Hz, 1H, H-7), 8.64 (dt, $J=6.7$, 1.5 Hz, 1H, H-2), 13.40 (bs, 1H, NH). Anal. calcd for $C_{14}H_8N_3Br$: C, 56.40; H, 2.70; N, 14.09. Found: C, 56.38; H, 2.75; N, 13.98.

Preparation of 8-nitroindolo[3,2-*c*]cinnolines (7h,i). To a solution of the indolo[3,2-*c*]cinnolines **7a**,**b** (2.3 mmol) in sulfuric acid (96%, 14 mL), a solution of potassium nitrate (2.3 mmol) in sulfuric acid (96%, 6 mL) was added dropwise, with stirring at 0°C. The reaction mixture was allowed to reach room temperature and kept under stirring overnight. It was then poured onto crushed ice and the solid was filtered off, air dried and recrystallized from ethanol. 8-Nitroindolo[3,2-*c*]cinnoline (**7h**) had mp >320°C (83% yield), IR: 3425 (broad NH), 1528 (NO_2) cm^{-1}; ^1H NMR ppm: 7.71 (dd, $J=6.8$, 1.6 Hz, 1H, H-1), 7.75 (dd, $J=6.8$, 1.6 Hz, 1H, H-4), 8.27 (dd, $J=7.8$, 1.8 Hz, 1H, H-9), 8.34 (dt, $J=6.8$, 1.6 Hz, 1H, H-3), 8.51 (dt, $J=6.8$, 1.6 Hz, 1H, H-2), 8.67 (d, $J=7.8$ Hz, 1H, H-10), 9.38 (d, $J=1.8$ Hz, 1H, H-7), 14.70 (bs, 1H, NH). Anal. calcd for $C_{14}H_8N_4O_2$: C, 63.64; H, 3.05; N, 21.20. Found: C, 63.77; H, 3.00; N, 21.14. 3-Chloro-8-nitroindolo[3,2-*c*]cinnoline (**7i**) had mp >320°C (70% yield), IR: 3403 (broad NH), 1548 (NO_2) cm^{-1}; ^1H NMR ppm: 7.72 (d, $J=2.0$ Hz, 1H, H-4), 7.73 (d, $J=7.0$ Hz, 1H, H-1), 8.31 (dd, $J=8.0$, 2.0 Hz, 1H, H-9), 8.47 (dd, $J=7.0$, 2.0 Hz, 1H, H-2), 8.83 (d, $J=2.0$ Hz, 1H, H-7), 9.31 (d, $J=8.0$ Hz, 1H, H-10), 13.14 (very bs, 1H, NH). Anal. calcd for $C_{14}H_7N_4O_2Cl$: C, 56.30; H, 2.36; N, 18.76. Found: C, 56.27; H, 2.42; N, 18.84.

Biology

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

Cells. Cell lines were from American Type Culture Collection (ATCC); bacterial and fungal strains were either clinical isolates (obtained from Clinica Dermosifilopatica, University of Cagliari) or collection strains from ATCC. H9/III_B, MT-4, and C8166 cells (grown in RPMI 1640 containing 10% foetal calf serum (FCS), 100 UI/mL penicillin G and 100 μg/mL streptomycin) were used for anti-HIV assays. Cell cultures were checked periodically for the absence of mycoplasma contamination with a MycoTect Kit (Gibco).

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 fifty ($CCID_{50}$)/mL.

Antiviral assays. Activity against the HIV-1 multiplication in acutely infected cells was based on inhibition of virus-induced cytopathogenicity in MT-4 cells.[6] 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. 20 μL of an HIV-1 suspension containing 100 $CCID_{50}$ were then added. After a 4 days incubation at 37°C, the number of viable cells was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method.[7,8] Cytotoxicity of compounds, based on the viability of mock-infected cells, as monitored by the MTT method, was evaluated in parallel with their antiviral activity.

Antiproliferative assays. Exponentially growing leukemia and lymphoma cells were resuspended at density of 1×10^5 cells/mL in RPMI containing serial dilutions of the test drugs. Cell viability was determined after 4 days at 37°C by the MTT method. Activity against cells derived from solid tumor was evaluated in exponentially growing cultures seeded at 5×10^4 cells/μL which were allowed to adhere for 16 h to culture plates before addition of the drugs. Cell viability was determined by the MTT method 4 days later.

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

Cytotoxicity assays. Peripheral blood lymphocites (PBL) from HIV-negative donors were obtained by separation on Fycoll–Hypaque gradients. After extensive washings, cells were resuspended (1×10^6 cells/mL) in RPMI-1640 with 10% FCS and incubated overnight.

For cytotoxicity evaluations in proliferating PBL cultures, non-adherent cells were resuspended at 1×10^6 cells/mL in growth medium, stimulated with PHA (2.5 μg/mL), for 1 day before dilution to 1×10^5 cells/mL in medium containing PHA (2.5 μg/mL), IL-2 (50 U/mL) and various concentrations of the test compounds. Viable cell number were determined 6 days later. Under these conditions, untreated PBL were able to undergo exponential growth up to four cell cycles, as determined by viable cell counts. For cytotoxicity evaluations in resting PBL cultures, non-adherent cell were resuspended at high density (1×10^6 cells/mL) and treated for as long as 3 days with the test compounds. Then, the cells were extensively washed to remove the inhibitors and were stimulated with PHA for 1 day before being diluted to 1×10^5 cells/mL in medium containing PHA and IL-2. Cell viability was determined after incubation at 37°C for 6 days.

Antibacterial assays. *Staphylococcus aureus*, group D *Streptococcus*, *Shigella* and *Salmonella* spp. were recent clinical isolates. Assays were carried out in nutrient broth, pH 7.2, with an inoculum at 10^3 cells/tube. Minimum inhibitory concentrations (MIC) were determined after incubation at 37°C for 18 h in the presence of serial dilutions of test compounds.

Antimycotic assays. Yeast inocula were obtained by properly diluting cultures incubated at 37°C for 30 h in Sabouraud dextrose broth to obtain 5×10^3 cells/mL. On the contrary, dermatophyte inocula were obtained from cultures grown at 37°C for 5 days in Sabouraud dextrose broth by finely dispersing clumps with a glass homogenizer before diluting to 0.05 OD_{590}/mL. Then, 20 μL of the above suspensions were added to each well of flat-bottomed microtiter trays containing 80 μL of medium with serial dilutions of test compounds, and were incubated at 37°C. Growth controls were visually determined after 2 days (yeast) or 3 days (dermatophytes). MIC was defined as the compound concentration at which no macroscopic signs of fungal growth were detected. The minimal germicidal concentrations (MBC or MFC) were determined by subcultivating in Sabouraud dextrose agar samples with no apparent growth.

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