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Structurally diverse 5-substituted pyrimidine nucleosides as inhibitors of *Leishmania donovani* promastigotes in vitro

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Abstract—The following structurally diverse 5-substituted-2′-deoxyuridine nucleosides displayed potent in vitro antileishmanial activity: 5-formyl, 5-(2,2,-dicyanovinyl)-, 5-(2-cyano-2-ethoxycarbonylvinyl), 5-(2-cyano-2-methoxycarbonylvinyl)-, 5-(2-amino-3-cyano-5-oxo-5,6,7,8-tetrahydro-4*H*-chromen-4-yl)- and related congeners, and the 5-(3-methyl-5-oxo-1-phenyl-4,5-dihydro-4*H*-pyrazol-4-ylidene) group.

Leishmania is protozoan parasitic human disease caused by any of 20 different species of *Leishmania* and is transmitted by the bite of some thirty species of phlebotomine sandflies.[1] The disease presents in four forms of increasing severity: diffuse cutaneous leishmaniasis, cutaneous leishmaniasis, mucocutaneous leishmaniasis, and visceral leishmaniasis (Kala Azar). At least 12 million individuals are infected worldwide with 2 million new cases occurring each year. Recently, HIV coinfection has added dangerous new dimensions to leishmaniasis.[1,2] First, visceral leishmaniasis accelerates the on set of AIDS from HIV, and second, HIV facilitates the spread of *Leishmania*. Presently, epidemics of cutaneous leishmaniasis exist in Afghanistan and Pakistan, and there is an epidemy of visceral infection in India and Sudan. In Brazil, visceral leishmaniasis is a reemerging disease.[1]

Four different drugs are used in the treatment of leishmaniasis including pentavalent antimony compounds (sodium stibogluconate and meglumine antimoniate), Amphotericin B and its liposomal form, and Miltefosine. All are toxic and resistance is well described for antimonials whereas resistance to amphotericin B and miltefosine is at risk, and only the latter can be administered orally.[1,2]

Peyron and co-workers[3] reported recently on a series of 5-heteroaryl-2′-deoxyuridine analogues, which possessed significant activity against *Leishmania donovani* promastigotes. The nature of the pyrimidine 5-substituents that provided active antileishmanial nucleosides included thiazole, thiophene, benzothiazole, and benzothiophene. The considerable steric size of these substituents and their attendant antileishmanial activities was of special interest as we have just reported on the synthesis and strong in vitro anti-orthopoxvirus activity of several pyrimidine nucleosides with diverse and often quite bulky 5-substituents.[4–6] Therefore, we have examined the ability of these new nucleosides to inhibit *L. donovani* growth in culture.

The starting point for this exploration (Fig. 1 and Table 1) of the antileishmanial activity of 5-substituted pyrimidine nucleosides may be considered as 5-formyl-2′-deoxyuridine (**1**), a potent inhibitor. This activity was lost upon 3′,5′-diacetylation of the hydroxyls (compound **2**). Conversion of the aldehyde to the nitrile to give 5-cyano-2′-deoxyuridine (**3**) also resulted in an inactive agent. Introduction of the dicyanoalkene motif to the pyrimi-

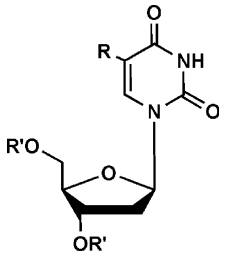

Figure 1. Generic structure of compounds evaluated in Table 1.

Keywords: Pyrazolone; Chromene; Cyanovinyl; Orthopoxvirus.
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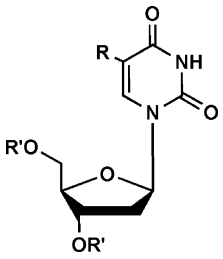
Table 1. Antileishmanial[a] activities of 5-substituted pyrimidine nucleosides[b]



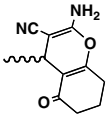
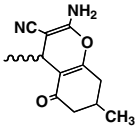
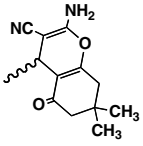
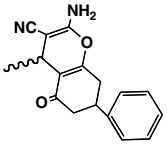
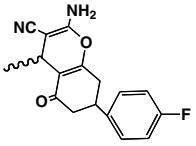
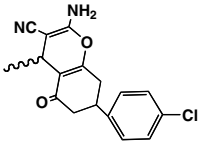
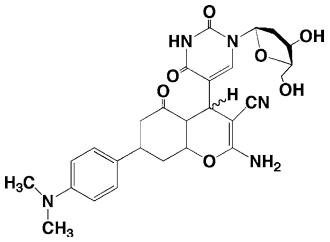
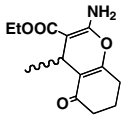
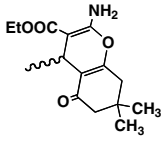
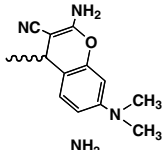
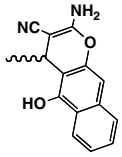
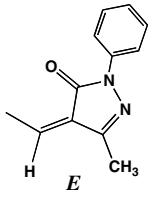
Compound	R	R′	IC_{50} (μM)
1	–CHO	H	0.9 ± 0.1
2	–CHO	Ac	>100
3	–CN	H	>100
4	–C(H)=C(CN)$_2$	H	1.5 ± 0.2
5	–C(H)=C(CN)$_2$	Ac	30.1 ± 2.8
6	–C(H)=C(H)CN (*E*, *Z*)	H	92
7	–C(H)=C(H)CN (*E*)	Ac	>100
8	–C(H)=C(H)CN (*Z*)	Ac	>100
9	–C(H)=C(CN)COOEt (*E*, *Z*)	H	1.9 ± 0.2
10	–C(H)=C(CN)COOEt (*E*, *Z*)	Ac	39.1 ± 4.2
11	–C(H)=C(CN)COOMe (*E*, *Z*)	H	7.3 ± 0.6
12		H	1.4 ± 0.1
13	Same as **12**	Ac	68.7 ± 6.9
14		H	9.4 ± 0.8
15	Same as **14**	Ac	>100
16		H	>100
17	Same as **16**	Ac	80.4 ± 7.2
18		Ac	85.9 ± 9.7
19		H	8.4 ± 0.9
20	Same as **19**	Ac	95.3 ± 8.5
21		H	>100
22	Same as **21**	Ac	80.2 ± 6.7

Table 1 (*continued*)

Compound	R	R′	IC_{50} (μM)
23		H	6.2 ± 0.7
24	Same as **23**	Ac	100
25		H	10.5 ± 1.5
26		H	7.2 ± 0.8
27	Same as **26**	Ac	>100
28		H	>100
29		H	>100
30	Same as **29**	Ac	>100
31		H	3.0 ± 0.2

[a] In vitro evaluation on promastigote forms. Promastigote forms of a *L. donovani* LV9 clone and the standard drugs pentamidine and amphotericin B were used for the assay. M-199 medium supplemented with 40 mM HEPES, 100 μM adenosine, 0.5 mg/ml hemin, 10% heat-inactivated fetal calf serum (hi-FCS), and 50 μg/ml gentamycin was added to each well of a 96-well microtiter plate. Serial drug dilutions were added to the wells. Then 2×10^5 promastigote forms of *L. donovani* in 100 μl were added to each well and the plate incubated at 27 °C (under a 5% CO_2 atmosphere) for 72 h. Each concentration was screened in triplicate. The viability of promastigotes was checked using the tetrazolium-dye (MTT) colorimetric method. The MTT cell proliferation assay is a colorimetric assay system, which measures the reduction of a tetrazolium component (MTT) into an insoluble formazan product by the mitochondria of viable cells. After incubation of the cells with the MTT reagent, a detergent solution was added to lyse the cells and solubilize the colored crystals. The samples were read using an ELISA plate reader at a wavelength of 570 nm. The amount of color produced was directly proportional to the number of viable cells. The results are expressed as the concentrations inhibiting parasite growth by 50% (IC_{50}) after a 3-day incubation period. Under these conditions, the IC_{50} of pentamidine was 6.6 ± 0.7 μM, the IC_{50} of amphotericin B was 0.10 ± 0.02 μM, and the IC_{50} of zidovudine, a pyrimidine nucleoside, was at 3.4 ± 0.3 μM.

[b] Nucleosides were obtained as described.[4–7]

dine base provided potently active compound **4**. As with compound **1**, acetylation of the sugar hydroxyls (compound **5**) resulted in a significant diminution (20-fold) of activity. Removal of one of the two cyano groups of **4** gave compound **6** with 60-fold decreased activity that was mirrored by the inactivity of its separate isomers in their acetylated forms (compounds **7** and **8**). One nitrile moiety could, however, be modified to an ethyl or methyl carboxyl ester to retain potency as shown with compounds **9**, **10**, and **11**. Within this series, the ethyl ester (compound **9**) was about fourfold more active than the methyl ester (compound **11**), and again, acetylation of the deoxyribose hydroxyls (compound **10**) effected a 20-fold decrease in antileishmanial potency.

Investigation of 'hypermodified' pyrimidine nucleosides, derived from synthetic hybridization of two different privileged drug scafffolds, provided a series of potent antileishmanial agents. This series begins with the simplest congener, 5-(2-amino-3-cyano-5-oxo-5,6,7,8-tetrahydro-4*H*-chromen-4-yl)-1-(2-deoxypento-furanosyl)pyrimidine-2,4(1*H*,3*H*)-dione (**12**), that displayed an antileishmanial IC_{50} of 1.4 μM. As before, sugar OH acetylation (compound **13**) caused a diminution (48-fold) of activity. The introduction of a single methyl group to the 7-position of the chromene ring (compound **14**) resulted in an activity decrease (6.7-fold) to an IC_{50} of 9.4 μM whereas an additional methyl group at chromene ring position 7 produced compound **16** and a complete loss of discernable activity. The deoxyribose diacetylation of compound **14** to yield compound **15** brought about a loss of bioactivity. Strangely, in the case of compound **16**, deoxyribose acetylation gave compound **17** that possessed some marginal albeit greatly reduced activity (IC_{50} = 80 μM) compared to the other structures.

Introduction of aromatic substituents at chromene ring position 7 had varying effects depending on the nature of the substituent. The diacetylated 7-phenyl substitution gave compound **18** with only marginal activity. In this case, the unsubstituted analogue was not available for comparison. However, placement of a *p*-fluorophenyl group at the 7-position of the chromene ring (**19**) resulted in good antileishmanial activity (IC_{50} = 8.4 μM) with diacetylation (**20**) causing an 11-fold reduction in activity. The similar substitution of the *p*-chlorophenyl group, however, gave a compound (**21**) devoid of activity with only some marginal activity in its diacetate (**22**). When a *p*-dimethylamino moiety was placed at chromene ring position 7, compound **23** was created with good antileishmanial activity (IC_{50} = 6.2 μM); however, the diacetylation of **23** to give compound **24** gave complete loss of activity.

The 3-cyano group of compound **12** could be replaced with the ethoxycarbonyl function (in compound **25**) with less than a 10-fold decrease in potency. Surprisingly, this same modification effected on the inactive dimethylchromene congener (compound **16**) gave rise to compound **26** with considerable activity (IC_{50} = 7.2 μM). As expected, the deoxyribose diacetate of **26** (compound **27**) was without activity.

Certain other major modifications to the chromene ring provided analogues without detectable antileishmanial activity. Thus, the benzopyran compound **28**, considered an analogue of compound **23**, was inactive. Likewise the benzochromene **29** (or its diacetate **30**) were also without activity. However, the methyl phenyl substituted pyrazolone-pyrimidine nucleoside (compound **31**) displayed potent antileishmanial activity (IC_{50} = 3 μM).

These results suggest several conclusions.

1. Although the active compounds were, aside from their pyrimidine nucleoside origin, of diverse structure, within reasonable related structures, there were distinct and dramatic structural dependencies. For instance, in the 5-vinylic congeners (compounds **4**, **6**, **9**, and **11**), it appeared that antileishmanial activity required two polar substituents on the terminal position of the alkene. Thus while compounds **4**, **9**, and **11** possessed significant activity, the monocyano analogue (as an *E*, *Z* mixture) was virtually inactive. Perhaps the most dramatic example of structure–activity dependence came from the unsubstituted, monomethyl, and dimethyl 2-amino-3-cyano-oxochromene series, compounds **12**, **14**, and **16**, wherein increasing methylation caused a decreasing antileishmanial activity. Finally, at least one structure–activity pattern was somewhat perplexing. Compound **19**, with its *p*-fluorophenyl substituent, possessed strong antileishmanial activity. This activity was abolished in the *p*-chlorophenyl congener **21**, but remarkably regained by the *p*-dimethylamino analogue **23**. The complete inactivity of the *p*-chlorophenyl analogue in view of the strong activities of the compounds bearing strongly electron-withdrawing fluorine and the strongly electron-donating dimethylamino moiety must be a matter for further investigation.

2. In all cases, when the parent nucleoside possessed strong antileishmanial activity, acetylation of the sugar hydroxyls caused a dramatic decrease in bioactivity. This would be most easily understood in terms of transporter recognition since the *Leishmania* promastigotes are known to possess a pyrimidine/purine transporter.[8] Indeed, five compounds were more active than the pyrimidine nucleoside zidovudine (AZT), and the most efficient was compound **1**, which was three times more active than the Zidovudine nucleoside reference compound.

3. Since most of the compounds described herein were chosen for this study based upon their surprising anti-orthopoxvirus activities, it is instructive to compare and contrast their activities against *Leishmania* promastigotes as opposed to orthopoxviruses (as represented by vaccinia virus (VV) and cowpox virus (CV)). Thus, 5-formyl-2′-deoxyuridine (compound **1**) as shown here possessed potent antileishmanial activity, but was devoid of any activity against VV and CV. The dicyanovinyl congener (**4**), potently active against *Leishmania*, was also potently active against CV and VV. In distinct contrast, however, the monocyanoanalogue, inactive against *Leishmania*, was very active against VV and CV. Compound **9**, active here against *Leishmania*, was also active against VV and CV. However, in stark contrast is the entire series of 7-substituted oxochromenes. While, as described above, there were distinct and considerable differences in these (compounds **12**, **14**, **16**, **19**, **21**, **23**) in their activity against *Leishmania*, against the orthpoxviruses, CV and VV, they were all potent inhibitors. Finally, the pyrazolone, compound **31**, was a potent inhibitor of both *Leishmania* and orthopoxviruses.

4. 5-Substituted pyrimidine nucleosides, including those with substantial steric size and bearing polar substituents, are a rich depot for further exploration as antileishmanial agents.

5. Finally, compounds having both antiviral and antileishmanial activities are of interest because in endemic

countries, patients are often affected by several pathogens, which synergize their deleterious effects on health.

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