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Anti-leishmanial activity of disubstituted purines and related pyrazolo[4,3-*d*]pyrimidines

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ABSTRACT

We report here results of screening directed to finding new anti-leishmanial drugs among 2,6-disubstituted purines and corresponding 3,7-disubstituted pyrazolo[4,3-*d*]pyrimidines. These compounds have previously been shown to moderately inhibit human cyclin-dependent kinases. Since some compounds reduced viability of axenic amastigotes of *Leishmania donovani*, we screened them for interaction with recombinant leishmanial cdc-2 related protein kinase (CRK3/CYC6), an important cell cycle regulator of the parasitic protozoan. Eighteen pairs of corresponding isomers were tested for viability of amastigotes and for inhibition of CRK3/CYC6 kinase activity. Some compounds (**9A**, **12A** and **13A**) show activity against amastigotes with EC_{50} in a range 1.5–12.4 μM. Structure–activity relationships for the tested compounds are discussed and related to the lipophilicity of the compounds.

Leishmaniasis encompasses a spectrum of human diseases caused by protozoan parasites belonging to the genus Leishmania. Designated a 'neglected disease' by the World Health Organization, it is found in more than 88 countries worldwide and where an estimated 350 million people are exposed to infection. The main diseases caused by these parasites include (i) cutaneous leishmaniasis, a self-limiting skin disease that leaves scars; (ii) mucocutaneous leishmaniasis, a debilitating, disfiguring, chronic disease of the nasopharynx and mucosal tissue; and (iii) visceral leishmaniasis, a fatal disease of the liver, spleen and bone marrow causing extensive morbidity and mortality. Recently, the leishmaniases appear to be spreading to regions previously free of these diseases due to multiple factors.[1]

Existing chemotherapeutics, such as pentavalent antimony, pentamidine and amphotericin B, show serious limitations and require intravenous injection, clinical supervision and hospitalization due to significant toxicity. Liposomal encapsulated amphotericin B exhibits lower toxicity and is very expensive, although a recent study suggests that costs may be significantly reduced by shortening the therapeutic regime.[2] In India, parasite resistance against pentavalent antimony drugs has become a serious problem, with >60% of the visceral leishmaniasis patients failing to respond to treatment.[3] Similar problems of parasite resistance appear in HIV/*Leishmania* co-infection patients not receiving highly active antiretroviral therapy, who tend to be refractory to treatment and frequently relapse.[4] Recently, miltefosine, the only oral drug for treating visceral leishmaniasis, was registered in India, Europe and South America.[5] Use of miltefosine in pregnant women is limited, however, due to teratogenic effects, and resistance to the drug develops easily in culture.[5]

Improved treatment protocols, such as combination therapy, are under investigation in an effort to optimize efficacy, reduce costs and prevent parasite resistance, but new drugs are urgently needed to expand the treatment options available for these diseases. Modern approaches are being employed that integrate genomic, proteomic and cellular analyses for developing novel and effective anti-leishmanial drugs. Rational drug design directed against parasite enzymes, such as dihydrofolate reductase, pteridine reductase or malate dehydrogenase, essential for proliferation or survival, has identified specific enzyme inhibitors, including trisubstituted pyrimidines, triazines and paullones.[6–11] Alternatively, parallels between parasites and cancer cells, including unlimited proliferation in the host, independence of exogenous growth

Abbreviations: CDK, cyclin-dependent kinase; CRK, cdc-2 related protein kinase; CYC, cyclin.

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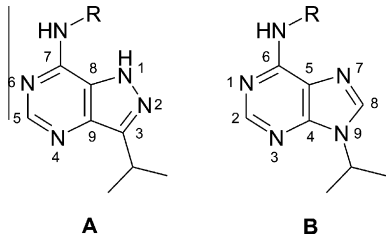

Figure 1. Structures of studied 3,7-disubstituted pyrazolo[4,3-*d*]pyrimidines (A) and 6,9-disubstituted purines (B).

factors and resistance to apoptosis, may provide new insights into drug development,[12] suggesting that anti-cancer drugs and compounds originally developed for oncological indications should be screened as potential leishmanicidal agents.[12,13] While such an approach led to the discovery of miltefosine, most anti-cancer drugs studied to date show only moderate anti-parasitic activity and have low selectivity indices, a major parameter in drug-toxicity evaluation.

Drug development for cancer has focused in recent years on protein kinase inhibitors. As parasite protein kinases frequently show limited homology to host enzymes and play important roles in regulating parasite proliferation, differentiation and survival, as well as virulence molecule expression and host protective responses, they have been proposed as targets for drug development. Indeed several leishmanial protein kinase families including cyclin-dependent kinase (CDK),[14,15] glycogen synthase kinase,[15] mitogen activated protein kinase[16] and cAMP dependent protein kinase[17] have been shown to be essential for parasite growth and survival.[7,17–19]

Analysis of the leishmanial kinome has identified 12 cdc-2 related protein kinases (CRKs) belonging to the CDK family and 11 cyclins (CYCs).[20] The large number of CRKs and CYCs may be related to the asynchronous replication of the nuclear and kinetoplast DNA during the cell cycle of these protozoan eukaryotes.[20,21] Several protein kinase inhibitors including flavopiridol,[22]

Table 1
Anti-leishmanial activity and CRK3/CYC6 inhibition activity of studied 3,7-disubstituted pyrazolo[4,3-*d*]pyrimidines (A series) and 6,9-disubstituted purines (B series)

Compound	R-substitution	*Leishmania donovani* axenic amastigotes inhibition		CRK3/CYC6 kinase inhibition	
		(%)[a]	EC_{50} (μM)	(%)[b]	IC_{50}[c] (μM)
1A	Benzyl	29.6 ± 0.1	68.6	24.5 ± 0.2	54.16
1B		19.9 ± 2.4	n.d.	18.5 ± 1.2	>100
2A	2-Hydroxybenzyl	87.4 ± 0.4	35.7	68.0 ± 2.1	11.91
2B		11.7 ± 2.7	n.d.	−2.8 ± 1.3	>100
3A	3-Hydroxybenzyl	25.4 ± 3.8	>100	35.8 ± 1.4	83.16
3B		6.5 ± 1.4	n.d.	8.8 ± 0.6	>100
4A	4-Methoxybenzyl	31.5 ± 3.3	83.0	36.0 ± 7.5	75.47
4B		15.1 ± 1.6	n.d.	−6.9 ± 9.8	>100
5A	3,4-Dimethoxybenzyl	8.4 ± 4.2	>100	19.9 ± 3.0	>100
5B		1.9 ± 0.5	n.d.	−2.3 ± 1.6	>100
6A	3-Hydroxy-4-methoxybenzyl	34.3 ± 2.4	94.0	46.8 ± 3.4	28.16
6B		8.8 ± 2.2	n.d.	11.2 ± 2.9	>100
7A	4-Hydroxy-3-methoxybenzyl	10.4 ± 2.4	>100	41.0 ± 1.8	100
7B		9.5 ± 1.1	n.d.	11.9 ± 0.1	>100
8A	2-Aminobenzyl	41.3 ± 2.2	>100	42.3 ± 0.5	58.64
8B		6.6 ± 4.0	n.d.	11.0 ± 6.3	>100
9A	Adamantan-1-yl	73.2 ± 0.0	1.22	93.8 ± 0.3	1.82
9B		72.0 ± 0.2	n.d.	66.1 ± 2.1	12.23
10A	3-Methylbut-2-en-1-yl	45.5 ± 2.3	85.0	32.5 ± 2.0	57.18
10B		0.04 ± 5.2	n.d.	3.2 ± 1.4	>100
11A	3-Fluorophenyl	66.6 ± 0.1	23.2	70.4 ± 1.8	14.56
11B		16.5 ± 0.7	n.d.	30.4 ± 0.7	49.92
12A	4-Fluorophenyl	75.8 ± 1.7	11.6	78.8 ± 0.4	6.8
12B		21.5 ± 1.1	n.d.	53.3 ± 6.1	22.84
13A	3-Chlorophenyl	73.3 ± 1.1	12.4	81.3 ± 2.3	9.86
13B		35.1 ± 3.3	n.d.	22.3 ± 4.9	>100
14A	2-Bromophenyl	40.7 ± 6.67	18.7	35.6 ± 3.4	16.13
14B		26.2 ± 3.2	n.d.	1.8 ± 5.0	>100
15A	2-Aminocyclohexyl	15.8 ± 4.1	>100	2.4 ± 3.6	>100
15B		4.5 ± 3.8	n.d.	−5.4 ± 3.4	>100
16A	4-Aminocyclohexyl	3.3 ± 1.4	>100	35.0 ± 1.6	>100
16B		1.0 ± 2.7	n.d.	26.4 ± 3.7	>100
17A	Furfuryl	16.6 ± 1.9	>100	28.5 ± 7.8	>100
17B		14.5 ± 3.0	n.d.	3.5 ± 1.7	>100
18A	Pentyl	27.3 ± 1.6	54.7	46.5 ± 2.7	>100
18B		22.6 ± 5.3	n.d.	14.8 ± 5.5	>100

n.d.—not determined; all values were determined by duplicate or triplicate assays.
[a] In the presence of 30 μM compound.
[b] In the presence of 15 μM ATP with 30 μM compound.
[c] In the presence of 15 μM ATP.

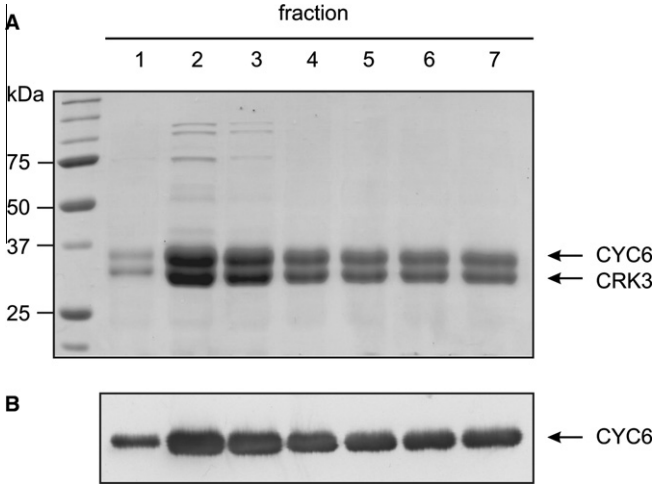

Figure 2. Elution profile of CRK3/CYC6 complex purification on a Co^{2+}-NTA column. (A) Ten microliter of each eluted fraction from Co^{2+}-NTA column was separated by SDS–PAGE and stained by a Coomassie Brilliant Blue for visualizing purity of the protein complex. Molecular masses of both proteins correspond to their predicted values (35.6 and 36.2 kDa for CRK3 and CYC6, respectively). (B) Detection of CYC6 by immunoblotting using an anti-His tag antibody. Lane numbering refers to the fraction number.

substituted purines,[14,15] paullones,[9,14] indirubins[9,14] and staurosporine derivatives,[14] have been screened for anti-leishmanial activity. Many of these compounds are also active on parasitic CRKs, and have been shown to block cell cycle and reduce parasite viability. One particular protein kinase, CRK3, is an essential enzyme for *Leishmania mexicana*,[22] and it has been shown, in complex with its binding partner CYC6,[23,24] to regulate the G2/M transition.[19] We describe a library of 6,9-disubstituted purines and corresponding 3,7-disubstituted pyrazolo[4,3-*d*]pyrimidines (Fig. 1) that have been previously shown to cause growth arrest, induce apoptosis in cancer cells, and inhibit human CDK1,[25] as leishmanicidal compounds targeting CRK3/CYC6 kinase.

We tested 18 isomeric pairs of purine and corresponding pyrazolo[4,3-*d*]pyrimidine (Table 1) for their ability to inhibit leishmanial CRK3 protein kinase activity and to kill axenic amastigotes of *Leishmania donovani*. Kinase inhibition assay was performed with CRK3/CYC6 complex (Fig. 2) and human histone H1.[28,29] Anti-amastigote activity was evaluated using a viability assay based on the reduction of alamarBlue.[26,27] All compounds were initially screened for anti-leishmanial activity at a single concentration (30 μM). The results are summarized in Table 1. Comparison of the activity of purine and pyrazolo[4,3-*d*]pyrimidine derivatives in the single point assay (Fig. 3A) clearly shows that pyrazolo[4,3-*d*]pyrimidines (A series) are markedly more potent inhibitors of CRK3/CYC6 activity than the corresponding purines. They were always more active with at least threefold greater activity observed for 12 compound pairs. More precise information about the CRK3/CYC6 inhibitory activity of the compounds was subsequently obtained from the dose–response kinase activity curves. Median IC_{50} values for pyrazolo[4,3-*d*]pyrimidines and purines were 57.9 and >100 μM, respectively (p <0.005, paired Wilcoxon test, two-sided). Analysis of structure–activity relationships in the A series (pyrazolo[4,3-*d*]pyrimidines) shows that the most potent inhibitors of CRK3/CYC6 either are highly lipophilic (adamantyl derivative **9A**, IC_{50} = 1.8 μM; halogenophenyl derivatives **11A–14A**, IC_{50} = 6.8–16.1 μM) or have 2-hydroxybenzyl group at the N^7-position (**2A**, IC_{50} = 11.9 μM) (Fig. 4). The positive inhibitory effect of *ortho*-substitution of benzyl groups on human CDK2 has been reported previously and explained by the stabilization of the active conformation of the inhibitor by an intramolecular hydrogen bond between *o*-hydroxy and the nitrogen in position 1 of the purine ring.[30] In contrast, activity of 2-aminobenzyl derivative (**8A**) was lower that of 2-hydroxybenzyl derivative (**2A**), but equaled to unsubstituted compound **1A**. On the other hand, most of the compounds showing no or limited activity in the kinase assay (IC_{50} >100 μM) belong to the most polar compounds in the set. These observations suggest that interaction between the N^7 (or N^6 for purines) substituent and the active site of CRK3 might be stabilized by hydrophobic interactions. In series B (purines), only adamantyl

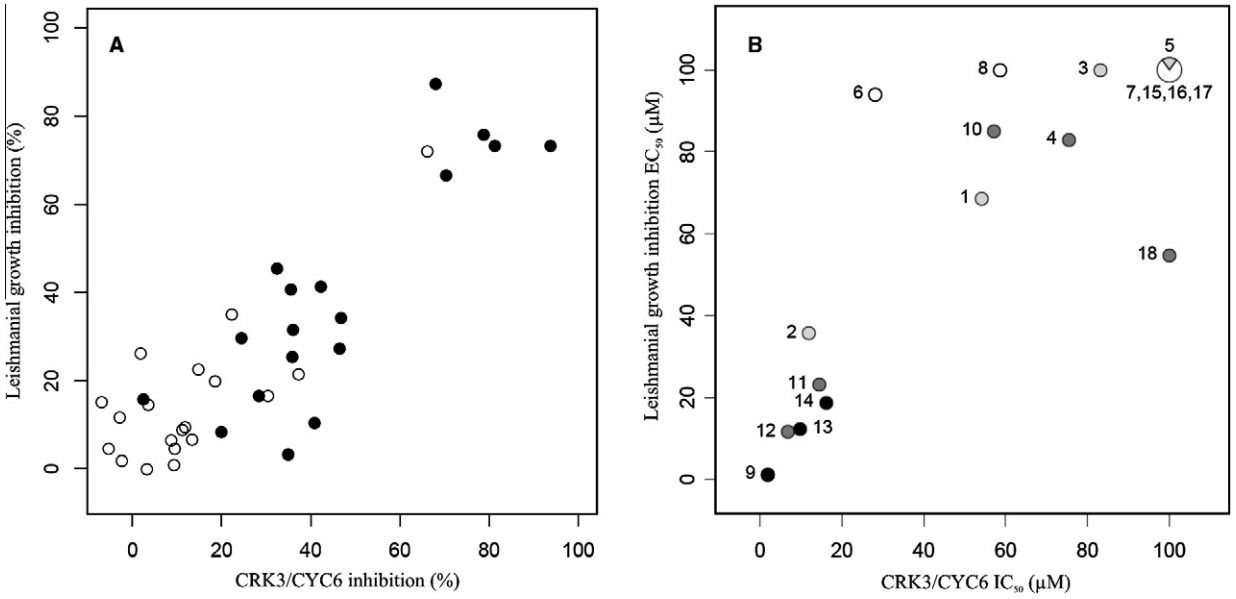

Figure 3. (A) Plot showing relationship between leishmanicidal activity and CRK3/CYC6 kinase inhibition (expressed as % inhibition) of studied 2,6-disubstituted purines (white circles) and corresponding 3,7-disubstituted pyrazolo[4,3-*d*]pyrimidines (black circles). Both determinations were carried out in at least duplicates using 30 μM of compound (Table 1). (B) Plot showing relationship between leishmanial axenic amastigote viability inhibition and CRK3/CYC6 kinase inhibition (IC_{50}) by 3,7-disubstituted pyrazolo[4,3-*d*]pyrimidines (A series). Circles correspond with lipophilicity (log *P*) of each compound, with log *P* values shown as shades of gray (white, 1.65–2.29; light gray, 2.29–2.94; dark gray, 2.94–3.58; black, 3.58–4.23). Numbering of the circles correspond with numbers of compounds listed in Table 1. Values of log *P* were calculated using ACD/PhysChem Suite software (version 12.0, ACD/Labs).

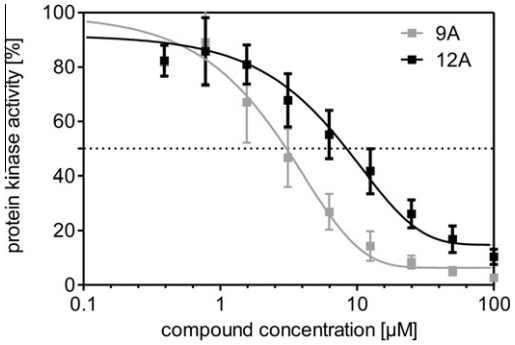

Figure 4. Inhibition of recombinant CRK3/CYC6 by the most effective compounds **9A** (grey curve) and **12A** (black curve).

purine **9B** (IC_{50} = 12.3 μM) and fluorophenyl derivatives **11B** and **12B** (IC_{50} = 49.9 and 22.8 μM, respectively) had IC_{50} values lower than the maximum concentration tested (100 μM). The observation that certain substitutions increase kinase inhibitory potency in both compound types suggests that pyrazolo[4,3-*d*]pyrimidines and purines might share a similar mode of binding to the CRK3/CYC6 complex.

Several studies using other human CDK inhibitors show that these compounds can block CRK activity and that this inhibition reduces parasite proliferation and viability.[14,15,22] Therefore, we screened all the compounds also for their ability to kill *Leishmania donovani* amastigotes, the form of the parasite responsible for disease. Anti-amastigote activity of the compounds is summarized in Table 1 and Figure 3, which clearly show that leishmanicidal activity reflects inhibition of the CRK3/CYC6 kinase. Average inhibitory effect of pyrazolo[4,3-*d*]pyrimidines at the single concentration of 30 μM was always higher than that of corresponding purine derivatives. Activity at least threefold greater was observed in the case of nine compound pairs. The most active pyrazolo[4,3-*d*]pyrimidines in terms of EC_{50} were adamantyl (**9A**), halogenophenyl (**12A**, **13A**) and 2-OH-benzyl derivatives (**2A**). Since these compounds are also the most potent inhibitors of CRK3/CYC6 in the datasets, the observed effect on parasite viability is probably mediated by CRK3/CYC6 inhibition in agreement with previous reports.[14] A link between the anti-amastigote activity of CDK inhibitors and leishmanial CRK inhibition was first suggested by experiments using flavopiridol, a pan-selective CDK inhibitor. This compound inhibits CRK3 kinase (IC_{50} ~100 nM), and it also has been shown to arrest the parasite's cell cycle.[22] Additional experiments have shown that trisubstituted purines and indirubin analogs (all known CDK inhibitors) that are potent CRK3 inhibitors are also effective in killing *Leishmania* in vitro.[14,15] Nevertheless, the possibility cannot be excluded that inhibition of another target contributes to the effect.

Finally, we note that our dataset demonstrates that the positive association between the activity and polarity in the group of pyrazolo[4,3-*d*]pyrimidines (Fig. 3B) was not limited to the viability assay but was also observed in the in vitro kinase assay. Our analysis demonstrates that the relationship between compound polarity and its cellular activity may in certain cases reflect the effect of polarity on the affinity for the binding site of a molecular target rather than its effect on the transport through biological membranes. Similar relationships between lipophilicity and anti-leishmanial activity of many diverse compounds have also been reported previously.[13,31]

In summary, the present study clearly shows that the series of 3,7-disubstituted pyrazolo[4,3-*d*]pyrimidines displaying moderate inhibition activity against the leishmanial CRK3/CYC6 protein complex also kills axenic amastigotes, thereby confirming previously published hypothesis.[22] These findings may not only provide chemical tools for basic studies on *Leishmania* biology, but they can also help to develop a new series of related compounds specifically directed against these parasites and having an improved therapeutic index, which will need further experiments with human cells and kinases.

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Supplementary data

Supplementary data (chemistry, experimental procedures, spectroscopic and analytical data, viability and kinase assays) associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2011.05.076.

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