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Authors: Glenn Burley, Allan John Bell Watson, Harry de Koning, Katherine Jones, Jack Robertson, Bela Bode, Fergus McWhinnie, Marzuq Ungogo, Mustafa Aldfer, and Leandro Lemgruber

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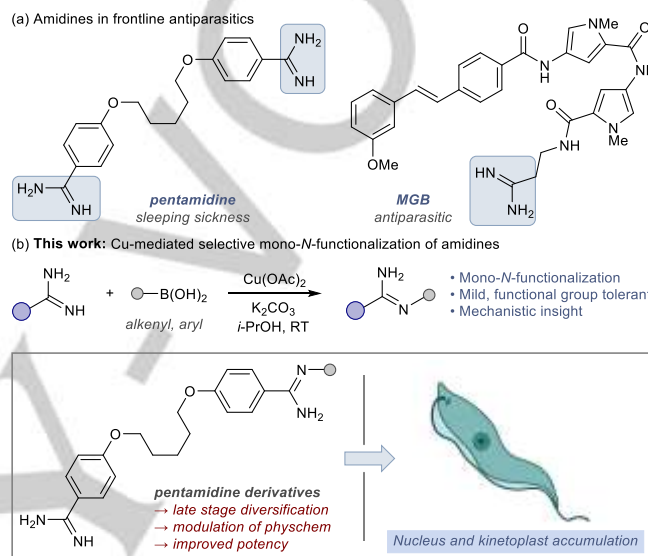
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Direct, late-stage mono-*N*-arylation of pentamidine: method development, mechanistic insights, and expedient access to novel antiparasitics against diamidine-resistant parasites

Jack Robertson,^[a] Marzuq A. Ungogo,^[b] Mustafa M. Aldfer,^[b] Leandro Lemgruber,^[c] Fergus S. McWhinnie,^[a] Bela E. Bode,^[d] Katherine L. Jones,^{[e]*} Allan J. B. Watson,^{[d]*} Harry P. de Koning,^{[b]*} Glenn A. Burley^{*[a]}

Abstract: A selective mono-*N*-arylation strategy of amidines under Chan–Lam conditions is described. During the reaction optimization phase, the isolation of a mononuclear Cu(II) complex provided unique mechanistic insight into the operation of Chan–Lam mono-*N*-arylation. The scope of the process is demonstrated, and then applied to access the first mono-*N*-arylated analogues of pentamidine. Sub-micromolar activity against kinetoplastid parasites was observed for several analogues with no cross-resistance in pentamidine and diminazene-resistant trypanosome strains and against *Leishmania mexicana*. A fluorescent mono-*N*-arylated pentamidine analogue revealed rapid cellular uptake, accumulating in parasite nuclei and the kinetoplasts. The DNA binding capability of the mono-*N*-arylated pentamidine series was confirmed by UV-melt measurements using AT-rich DNA. This work highlights the potential to use Chan–Lam mono-*N*-arylation to develop therapeutic leads against diamidine-resistant trypanosomiasis and leishmaniasis.

Amidines are essential functional groups used throughout medicinal chemistry.^[1] The versatility of this motif arises from their ability to form strong, bifurcated hydrogen bonds and electrostatic interactions with a range of hydrogen bond acceptors and conjugate bases.^[2] In addition, modulating the basicity of the amidine group ($pK_{\text{aH}} \sim 13\text{--}14$) alters the overall physicochemical properties, potency, and selectivity of amidine-containing



Scheme 1. (a) Amidines in antiparasitic agents. (b) Selective amidine mono-*N*-arylation and application to development of pentamidine antiparasitics. MGB = minor groove binder.

therapeutics.^[3] A prominent exemplar of this approach is the development of the DNA-binding antiparasitic pentamidine for the treatment of early-stage *Trypanosoma brucei gambiense*-related human African trypanosomiasis (HAT) or sleeping sickness,^[4] AIDS-related pneumocystis pneumonia, and leishmaniasis (Scheme 1a).^[5] The broader importance of diamidine antiparasitics is further exemplified by their use as the main treatment for animal African trypanosomiasis (AAT), caused by the related parasite *Trypanosoma congolense*, which has a billion-dollar adverse impact on emerging economies.^[6]

Poor oral bioavailability and the emergence of pentamidine-resistant strains has spurred the exploration of new DNA-binding analogues (Scheme 1a).^[7] Modification of the essential amidine moiety is a nascent strategy to by-pass resistance mechanisms in these parasites by modulating the affinity of diamidines for key drug transporters in *T. brucei*.^[8] [e.g., TbAT1/P2 aminopurine transporters,^[9] High Affinity Pentamidine Transporter (HAPT)^[10]].^[11] However, expedient synthetic access to mono-*N*-arylated amidines has been hampered by the lack of robust synthetic methodology suitable for structure-activity relationship profiling.^[12] existing Pinner-style methodology^[13] is unsuited to this challenge due to forcing reaction conditions limiting functional group tolerance, and the lack of chemoselective control leading to competing di/tri-*N*-arylation.^[14] By virtue of readily-available starting materials, Pd-^[15] and Cu-catalyzed cross couplings (e.g.,

[a] Dr J. Robertson, Dr F. S. McWhinnie, Prof. G. A. Burley
Department of Pure and Applied Chemistry
University of Strathclyde
295 Cathedral Street, Glasgow, G1 1XL (UK)
E-mail: glenn.burley@strath.ac.uk

[b] Mr M. A. Ungogo, Mr M. M. Aldfer, Prof. H. P. de Koning
Institute of Infection, Immunity, and Inflammation
College of Medical, Veterinary, and Life Sciences
University of Glasgow, Glasgow, G12 8TA (UK)
Email: harry.de-koning@glasgow.ac.uk

[c] Dr L. Lemgruber
Glasgow Imaging Facility
Institute of Infection, Immunity, and Inflammation
College of Medical Veterinary and Life Sciences
University of Glasgow, Glasgow, G12 8TA (UK)

[d] Dr B. E. Bode, Dr A. J. B. Watson
EaStCHEM, School of Chemistry
University of St Andrews
North Haugh, St Andrews, Fife, KY16 9ST (UK)
Email: aw260@st-andrews.ac.uk

[e] Dr K. L. Jones
GlaxoSmithKline
Medicines Research Centre
Gunnels Wood Road, Stevenage, Hertfordshire, SG1 2NY (UK)

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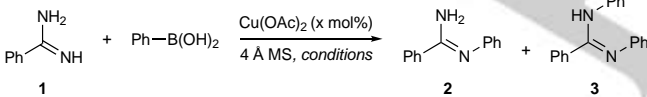
Ullmann,^[3c, 16] Chan-Lam^[17]) provide the opportunity to selectively access mono-*N*-functionalised amidine analogues.

Here we report a mild and chemoselective approach to exemplify amidine mono-*N*-arylation *via* the optimization of a Chan-Lam strategy (Scheme 1b). We expand the substrate scope and demonstrate its application to access, for the first time, mono-*N*-arylated pentamidines as anti-parasitic leads for treatment of HAT, AAT, and *leishmaniasis*.^[18]

A prominent challenge of amidine functionalization is controlling mono- vs. di/tri-*N*-arylation.^[17b, 19] Although selective amidine *N*-arylation strategies have been reported, these are embedded within cascade processes alongside C–H activation events.^[17b, 20] As such, the identification of reaction variables which influence mono- vs. di/tri-*N*-arylation was first established.

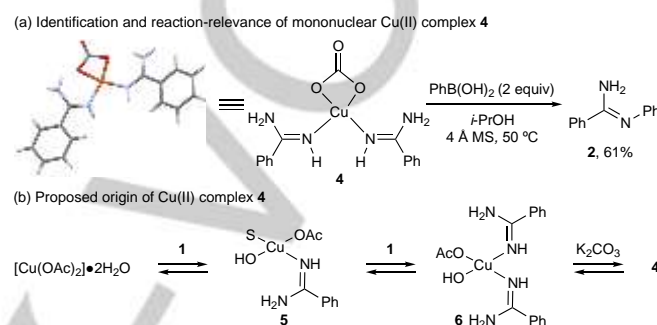
Under standard Chan-Lam conditions,^[21] a model system using benzamidine (**1**) and PhB(OH)₂ gave 73% overall yield and ca. 4:1 selectivity in favor of the mono- (**2**) vs. *N,N*-diarylated product **3** (Entry 1, Table 1). Using an excess of PhB(OH)₂ led to 82% total yield with ca. 9:1 selectivity for **3** (Entry 2), which suggested selective mono-*N*-arylation vs. *N,N*-diarylation is possible by controlling reagent stoichiometry. No tri-*N*-functionalization products were observed. Vantourout's B(OH)₃-based conditions were less effective (Entry 3).^[22] Li's NaOPiv-based conditions provided a high conversion and ca. 40:1 selectivity for **2** (Entry 4);^[17b] however, further exploration of these conditions did not improve the conversion to **2** (Table S5). The observation of a pronounced base effect led to a focused screen and identification of K₂CO₃ as the optimal additive (Entries 5–9). This screen ultimately provided selective mono-*N*-arylation set of conditions using 20 mol% Cu(OAc)₂ giving **2** in 81% isolated yield and without any observable generation of **3** (Entry 10).

Table 1. Reaction development.^[a]

		
Entry	Conditions	2/3 (%) ^[b]
1 ^[c]	Cu(OAc) ₂ (100 mol%), Et ₃ N, CH ₂ Cl ₂ , RT, 16 h	59/14
2 ^[c,d]	Cu(OAc) ₂ (100 mol%), Et ₃ N, CH ₂ Cl ₂ , RT, 16 h	8/74
3	Cu(OAc) ₂ (100 mol%), B(OH) ₃ , CH ₂ Cl ₂ , RT, 16 h	45/11
4 ^[d]	Cu(OAc) ₂ (100 mol%), NaOPiv, DMF, 50 °C, 16 h	81/2
5	Cu(OAc) ₂ (100 mol%), K ₂ CO ₃ , CH ₂ Cl ₂ , RT, 16 h	40/8
6	Cu(OAc) ₂ (100 mol%), K ₂ CO ₃ , MeOH, RT, 16 h	59/2
7 ^[c]	Cu(OAc) ₂ (100 mol%), K ₂ CO ₃ , <i>i</i> -PrOH, RT, 2 h	83/5
8	Cu(OAc) ₂ (50 mol%), K ₂ CO ₃ , <i>i</i> -PrOH, 50 °C, 24 h	76/2
9 ^[c]	Cu(OAc)₂ (20 mol%), K₂CO₃, <i>i</i>-PrOH, 50 °C, 24 h	81/0

^[a] Using 2:1 **1**:PhB(OH)₂ and 2 equiv of additive unless noted. ^[b] Determined by HPLC analysis using standard concentration curves of **2** and **3**. ^[c] Isolated yield on 1 mmol scale. ^[d] **1**:PhB(OH)₂ = 1:2. ^[e] **1**:PhB(OH)₂ = 1:1.2.

In contrast to our experience with other Chan-Lam processes,^[22–23] reactions using **1** and K₂CO₃ produced a purple precipitate within the first 5 minutes of the reaction (Figure S3). X-ray crystallographic analysis revealed a C₂-symmetric mononuclear Cu(II) complex **4** (Scheme 2a) consisting of a bidentate carbonate ligand and two monodentate benzamidines. This unique complex suggests that K₂CO₃ plays a dual role in this system, acting both as a ligand and base. A dual ligand/base role has been speculated in some Cu(OAc)₂-based Chan-Lam reactions;^[24] however, structural information has been limited to the observation of a single tetranuclear Cu(II) complex.^[22]



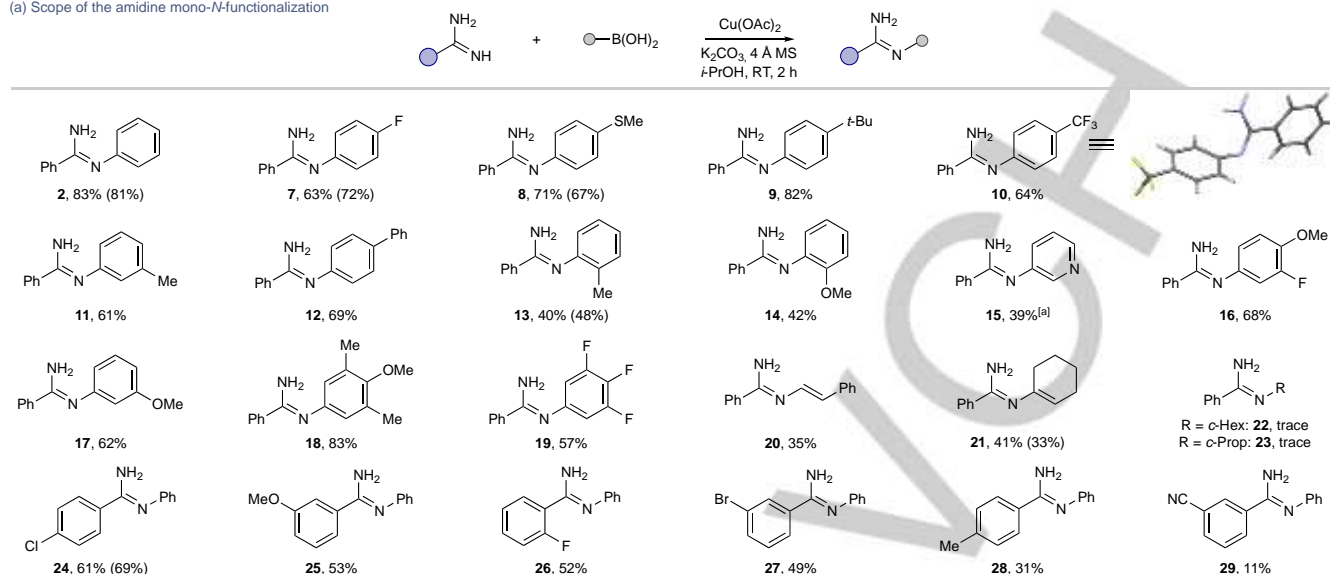
Scheme 2. (a) Structure of Cu(II) complex **4**. (b) Chan-Lam bond formation using complex **4**. (c) Proposed mechanistic origin of complex **4**. S = solvent.

Exposing **4** to PhB(OH)₂ in *i*-PrOH afforded **2** in 61% yield, suggesting **4** is a reaction-relevant intermediate (Scheme 2a). The role of **4** was further investigated by EPR analysis of reaction mixtures. No EPR signals were observed when [Cu(OAc)₂]₂•2H₂O was treated with K₂CO₃ or PhB(OH)₂. In contrast, a paramagnetic species was observed upon addition of **1** (Figure S2). This suggests that **1** is responsible for paddlewheel denucleation, consistent with mechanistic proposals for the Chan-Lam amination.^[22, 25] Complex **4** could form after paddlewheel denucleation *via* ligand exchange processes at, for example, putative Cu(II) complexes **5** and **6** (Scheme 2b).

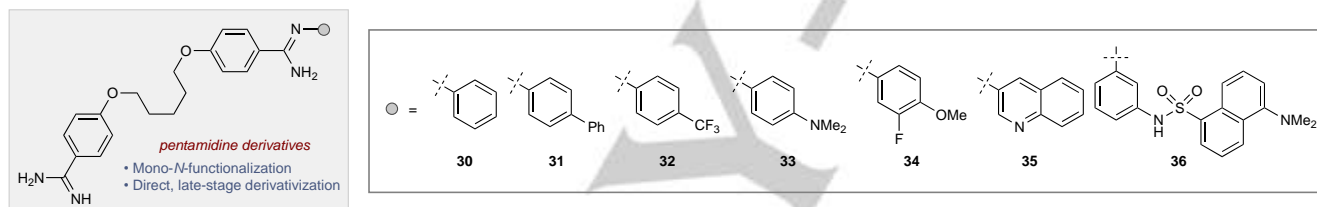
Supplementary to our main aim of using a Chan-Lam strategy for the late-stage mono-*N*-arylation of pentamidine, the generality of the approach was assessed using a range of amidines and arylboronic acids, all of which exclusively formed mono-*N*-arylated products (**2–29**, Scheme 3a, Table S6). The use of 20 mol% Cu(OAc)₂ was comparable to stoichiometric Cu(OAc)₂; however, for expediency, the latter was used across the series based on the faster reaction time (see Table 1, Entries 7 and 9) with comparable yields afforded for both processes (see yields for catalytic reactions in parentheses).

Both mono- and bisamidines are known Cu-chelators,^[26] which could hamper the wider utility of this approach to mono-*N*-arylate pentamidine for structure-activity-relationship (SAR) profiling. Indeed, standard Chan-Lam conditions failed to deliver any *N*-arylation of pentamidine. Gratifyingly, our reaction conditions were leveraged to directly access a series of exclusively mono-*N*-arylated pentamidines (**30–36**, Scheme 3b) in a single step from pentamidine.^[5b, 27]

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(a) Scope of the amidine mono-*N*-functionalization

(b) Library of monoarylated pentamidine derivatives

**Scheme 3.** (a) Scope of mono-*N*-arylation. Yields in parentheses for reactions at 50 °C for 24 h using 20 mol% Cu(OAc)_2 . (b) Library of monoarylated pentamidine derivatives. ^[a] Isolated as the corresponding TFA salt.**Table 2.** Activity of mono-*N*-aryl pentamidine analogues **30–36** against kinetoplastid parasites.

Entry	Compound	<i>T. b. brucei</i>				<i>T. congolense</i>				<i>L. mexicana</i>
		EC ₅₀ (μM; n = 3)		RF	t-test	EC ₅₀ (μM; n = 3)		RF	t-test	EC ₅₀ (μM; n = 4)
		WT	B48			WT	DA-Res			
1	30	1.10±0.07	1.31±0.02	1.19	0.042	2.94±0.10	3.26±0.01	1.11	0.037	12.7±0.7
2	31	0.91±0.02	0.86±0.02	0.94	0.10	n.d.	n.d.	---	---	2.12±0.07
3	32	0.65±0.03	0.37±0.006	0.57	0.0007	1.26±0.03	1.56±0.06	1.23	0.012	9.71±0.56
4	33	0.19±0.003	0.28±0.006	1.47	0.0002	0.64±0.02	0.75±0.003	1.23	0.0008	0.66±0.04
5	34	0.51±0.004	0.51±0.02	1.01	0.82	n.d.	n.d.	---	---	>20
6	35	1.09±0.01	0.82±0.01	0.75	0.0001	n.d.	n.d.	---	---	>20
7	36	3.04±0.02	2.98±0.04	0.98	0.23	6.69±0.11	9.29±0.17	1.39	0.0002	7.54±0.59
8	PMD	0.0024±0.0002	0.28±0.002	116	3.5x10 ⁻⁸	1.31±0.02	1.42±0.01	1.08	0.013	2.04±0.04
9	DA	0.082±0.006	0.89±0.05	10.8	0.0001	0.27±0.008	1.71±0.02	6.4	2x10 ⁻⁷	n.d.

WT = wild-type, PMD = pentamidine, DA = diminazene aceturate, DA-Res = diminazene-resistant cell line, RF = resistance factor being the ratio of EC₅₀ (resistant line) over EC₅₀(WT). Statistical difference between WT and resistant pairs of cell lines was established using a Student unpaired two-tailed t-test.

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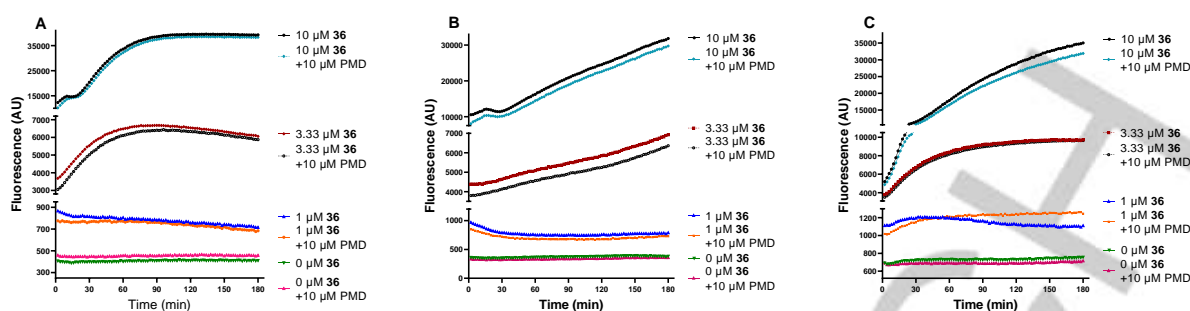


Figure 1. Real time fluorescence development of **36** with bloodstream forms of WT *T. b. brucei* (A), bloodstream forms of WT *T. congolense* (B) and promastigotes of *L. mexicana* (C). Cells were incubated for 3 h in the presence of **36** at 0, 1, 3.3 or 10 μM, in the presence or absence of 10 μM pentamidine. Measurements were taken at 2-minute intervals. A.U., artificial units of fluorescence intensity.

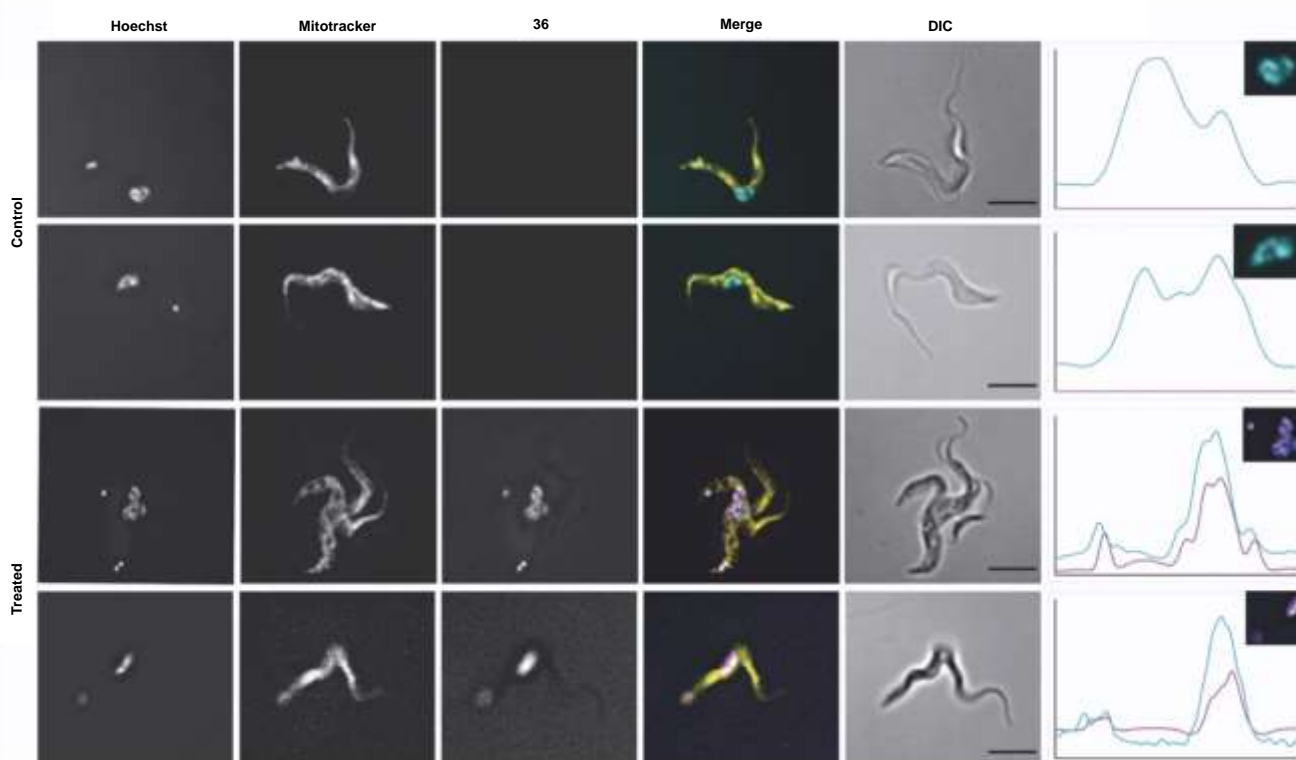


Figure 2. Selected immunofluorescence images of *T. brucei* labelled with a nuclear marker (Hoechst 33342), a dye for the mitochondrion (MitoTracker Green), and with **36** (30 min exposure, [10 μM]) or not (control). Charts show the intensity levels of the DAPI and red filter channels measured in the nuclei and kinetoplast. Scale bars are 5 μm.

This focused series covered analogues ranging from unsubstituted (**30**), mono-substituted (**31–33**) and disubstituted aromatics (**34**), a quinolone (**35**), and the bulky fluorescent analogue (**36**) suitable for cellular uptake studies.

Compounds (**30–36**) were then tested for activity against three kinetoplastid parasite species that are commonly treated with pentamidine (*T. brucei*, *L. mexicana*) or diminazene aceturate (*T. congolense*) (Table 2).^[28] EC₅₀ values ranged from 0.19 μM to 1.1 μM, with **33** exhibiting the greatest potency followed by **34**. Indeed, for *T. brucei*, the SAR was quite flat, with quinoline (**35**) and biphenyl (**31**) analogues displaying similar EC₅₀s to the phenyl analogue (**30**).

The compounds were also tested against the related kinetoplastid parasites *L. mexicana* and *T. congolense*. Here, **33** displayed >2-fold and >3-fold higher activity compared to pentamidine against *T. congolense* and *L. mexicana*, respectively. The SAR profile was considerably less flat than for *T. brucei*, with **33** being 4.6-fold and 19.2-fold more active than **30** against *T. congolense* and *L. mexicana*, respectively.

The potential for cross-resistance with diamidines and melaminophenyl arsenicals (MPAs; including melarsoprol, cymelarsan) is particularly problematic in the development of next-generation amidine antiparasitics.^[4] Resistance likely emerges from active drug transport [e.g., TbAT1/P2 aminopurine

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transporter;^[29] High Affinity Pentamidine Transporter (TbAQP2)] into the parasite's interior.^[30] Having studied the SAR of the transporter-diamidine interactions, we hypothesized that mono-*N*-arylation of the pentamidine scaffold would abolish their ability to act as substrates for the *T. brucei* drug transporters, and therefore would not display cross-resistance with pentamidine and MPAs.^[31] To test this hypothesis, **30–36** were also tested against a *T. b. brucei* clonal line, B48,^[32] lacking both the TbAT1/P2 and HAPT1/AQP2 transporters, and against a diminazene-resistant *T. congolense*^[11c] clone. Although minor differences in drug sensitivity were observed ($\pm 50\%$ of WT EC_{50}), these variations were trivial compared to the resistance levels to pentamidine (116-fold) and diminazene (6.4-fold), respectively. We therefore conclude that mono-*N*-arylated pentamidine analogues are not cross-resistant with un-substituted diamidines, arising from differences in their mechanism of uptake.

To gain further insights into the uptake of the mono-*N*-arylated pentamidines, the fluorescent analogue (**36**) was used to monitor uptake in each of the three parasite species in real-time (Figure 1). In all three species, **36** was taken up rapidly (3.3 μ M and 10 μ M), consistent with an $EC_{50} \approx 3 - 7 \mu$ M against the kinetoplastid species (Table 2). The rate of uptake of **36** was dose-dependent and showed an approximately 2.3 - 10-fold higher rate at 10 μ M [**36**] than for 3.3 μ M after 30 min (Figure 1). This shows that the uptake mechanism was not saturable in the lower μ M range, unlike TbAT1/P2 and HAPT/TbAQP2, which display K_m values of 0.26 ± 0.03 and $0.036 \pm 0.06 \mu$ M, respectively, for pentamidine.^[30] Moreover, the rate of uptake of **36** was not substantially different in the presence of 10 μ M pentamidine (within 10% in all cases), clearly indicating that uptake did not involve a high affinity pentamidine transporter. Taken collectively, these data show that mono-*N*-arylated analogues such as **36** evade known pentamidine transporters TbAT1 and TbAQP2 in *T. brucei*.

One of the putative mechanisms of action of pentamidine is the inhibition of replication *via* binding to A/T-rich sequences of duplex DNA.^[33] Indeed, selective accumulation of **36** to the nucleus and kinetoplast of *T. brucei* was observed, as confirmed by co-localization with Hoechst 33342 (Figure 2), consistent with DNA binding. UV melt analyses were undertaken to explore the ability of **33** and **36** to stabilize DNA duplexes relative to pentamidine (Table S7, Figure S4). A 7.0 °C stabilization of an A-tract duplex was observed for **33**, relative to a 6.0 °C stabilization for pentamidine. Although a reduced level of duplex stabilization was observed using **36** for the same sequence (ΔT_m 4.6 °C), both analogues exhibited a similar binding bias for an A-tract duplex relative to a duplex containing an alternating A-T sequence.^[34] These data indicate that the likely mechanism of action of these analogues is *via* binding to DNA duplexes with a selectivity profile similar to that observed for pentamidine. The stronger DNA binding of **33** correlated with its stronger anti-kinetoplastid activity compared to **36**.

In summary, we have developed a general approach to prepare mono-*N*-arylated amidines from mono- and bisamidine substrates based on Chan–Lam cross-coupling methodology. During our optimization phase, key mechanistic insights into Cu intermediates pertinent to the Chan–Lam reaction were identified, and the procedure was broadly applicable to the formation of mono-*N*-arylated substrates. This methodology was used to

directly prepare mono-*N*-arylated analogues of pentamidine, which displayed promising *in vitro* activity against three species of kinetoplast parasites of clinical and veterinary importance. Most importantly, the mono-*N*-arylated analogues were not cross-resistant with pentamidine and diminazene, bypassing known drug transporters. In addition, **36** accumulated rapidly in all three kinetoplastid species and localized to the parasite nucleus and kinetoplast, consistent with a putative mechanism of action being a DNA minor groove binder. These findings highlight that the potential utility of mono-*N*-arylation of diamidines, and potentially their expansion to guanidine analogues,^[35] as an emerging class of therapeutic agents against neglected parasitic diseases.

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Keywords: antiparasitics • amidine • arylation • copper • medicinal chemistry

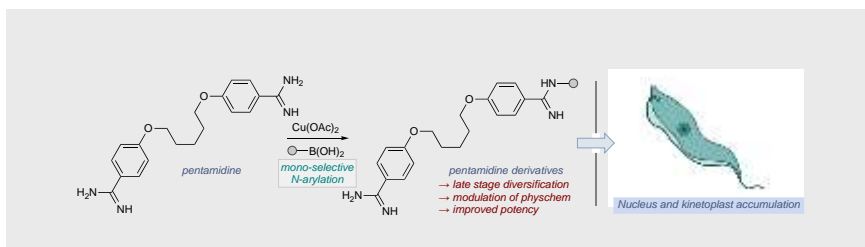
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Jack Robertson,^[a] Marzuq A. Ungogo,^[b]
Mustafa M. Aldfer,^[b] Leandro
Lemgruber,^[c] Fergus S. McWhinnie,^[a]
Bela E. Bode,^[d] Katherine L. Jones,^[e]*
Allan J. B. Watson,^[d]* Harry P. de
Koning,^[b]* Glenn A. Burley^{*[a]}

Direct, late-stage mono-N-arylation of pentamidine: method development, mechanistic insights, and expedient access to novel antiparasitics against diamidine-resistant parasites

A selective mono-N-arylation strategy of amidines under Chan–Lam conditions is described. During the reaction optimization phase, the isolation of a mononuclear Cu(II) complex provided unique mechanistic insight into the operation of Chan–Lam mono-N-arylation. The scope of the process is demonstrated, and then applied to access the first mono-N-arylated analogues of pentamidine. Sub-micromolar activity against kinetoplastid parasites was observed for several analogues with no cross-resistance in pentamidine and diminazene-resistant trypanosome strains and against *Leishmania mexicana*. A fluorescent mono-N-arylated pentamidine analogue revealed rapid cellular uptake, accumulating in parasite nuclei and the kinetoplasts. The DNA binding capability of the mono-N-arylated pentamidine series was confirmed by UV-melt measurements using AT-rich DNA. This work highlights the potential to use Chan–Lam mono-N-arylation to develop therapeutic leads against diamidine-resistant trypanosomiasis and leishmaniasis.