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Antimalarial activity of novel 4-cyano-3-methylisoquinoline inhibitors against *Plasmodium falciparum*: design, synthesis and biological evaluation†

Melissa J. Buskes,‡[a] Katherine L. Harvey,‡[b,c] Benjamin J. Richards,[a] Robabeh Kalhor,[a] Rebecca M. Christoff,[a] Chamodi K. Gardhi,[a] Dene R. Littler,[d] Elliott D. Cope,[b] Boris Prinz,[b] Greta E. Weiss,[b] Nathan J. O'Brien,[a] Brendan S. Crabb,[b,c,d] Leslie W. Deady,[a] Paul R. Gilson[b,d] and Belinda M. Abbott*[a]

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Central to malaria pathogenesis is the invasion of human red blood cells by *Plasmodium falciparum* parasites. Following each cycle of intracellular development and replication, parasites activate a cellular program to egress from their current host cell and invade a new one. The orchestration of this process critically relies upon numerous organised phospho-signaling cascades, which are mediated by a number of central kinases. Parasite kinases are emerging as novel antimalarial targets as they have diverged sufficiently from their mammalian counterparts to allow selectable therapeutic action. Parasite protein kinase A (*Pf*PKA) is highly expressed late in the cell cycle of the parasite blood stage and has been shown to phosphorylate a critical invasion protein, Apical Membrane Antigen 1. This enzyme could therefore be a valuable drug target so we have repurposed a substituted 4-cyano-3-methylisoquinoline that has been shown to inhibit rat PKA with the goal of targeting *Pf*PKA. We synthesised a novel series of compounds and, although many potently inhibit the growth of chloroquine sensitive and resistant strains of *P. falciparum*, they were found to have minimal activity against *Pf*PKA, indicating that they likely have another target important to parasite cytokinesis and invasion.

Introduction

Malaria represents a major global health challenge causing widespread morbidity and mortality and placing a huge medical, social and economic burden upon resource-poor populations. Nearly half of the world's population is at risk of infection and the World Health Organisation estimates that over 500 000 deaths and nearly 200 million clinical cases arise each year.[1] Although these figures indicate there has been progress in recent years in reducing the burden of disease much remains to be done to achieve eventual elimination and eradication. Despite significant efforts to reduce the disease's impact, major obstacles to overcome are a lack of an effective vaccine and increasing resistance to available therapies. New antimalarial therapeutics are therefore urgently needed but their design requires the identification and characterisation of novel potential drug targets.[2,3]

Malaria parasites have a complex life cycle that encompasses a succession of developmental stages across different cells types and host species, and a detailed understanding of the molecular mechanisms that underpin key events will be essential to inform the development of therapeutic strategies.[4]

[a]*Department of Chemistry and Physics, La Trobe Institute for Molecular Science, La Trobe University, Melbourne, Victoria 3086, Australia. E-mail: b.abbott@latrobe.edu.au; Tel: +61 3 94792520*
[b]*Centre for Biomedical Research, Burnet Institute, Melbourne, Victoria 3004, Australia*
[c]*Department of Microbiology and Immunology, Peter Doherty Institute, University of Melbourne, Melbourne, Victoria 3010, Australia*
[d]*Monash University, Melbourne, Victoria 3800, Australia*

†Electronic supplementary information (ESI) available: Copies of the ^{1}H and ^{13}C NMR spectra of compounds **9–50**. Counter screen of compounds **4**, **12**, **13**, **23** and **25**. Video S1. Live cell image sequence of a *Plasmodium falciparum* schizont-stage parasite treated with 10 μM compound **25** showing egressing merozoites and their attempts to invade nearby erythrocytes. Some invasions were successful (white arrows) whilst some failed to penetrate (pink arrow) or could penetrate but not reseal their erythrocyte host (red arrow). Video S2. Live cell image sequence of a *Plasmodium falciparum* schizont-stage parasite treated with 10 μM compound **25** showing egressing merozoites and their attempts to invade nearby erythrocytes. Some invasions were successful (white arrows) whilst some failed to penetrate (pink, yellow, orange and green arrows) or could penetrate but not reseal their erythrocyte host (red and blue arrows). See DOI: 10.1039/c5ob02517f

‡These authors contributed equally.

Proliferation, and specifically invasion, requires the co-ordinated modulation of specific gene sets and strict control of the cellular machinery. Malaria parasites regulate these processes by several means including phospho-signaling, a universally described intracellular regulatory mechanism.[5–7] Several malarial protein kinases are now known to be essential for parasite growth, replication and dissemination and these have arguably diverged sufficiently from their host counterparts to represent selectable therapeutic targets and a route to the next generation of antimalarials.[8,9]

The cyclic AMP-dependent protein kinase (PKA) of *P. falciparum*, the causative agent of the most lethal form of human malaria, represents an attractive target for the development of drugs against this species.[10,11] In most organisms PKA exists as a heterotetramer comprising of two regulatory subunits that bind and inhibit the activity of two catalytic subunits, but *Pf*PKA has only a single regulatory and catalytic subunit. Binding of two cyclic AMP (cAMP) molecules to the 51 kDa regulatory subunit causes disassociation and activation of the 40 kDa catalytic subunit that phosphorylates substrates using ATP as a phosphate donor.[11–13] Despite its relatively high sequence homology with that of *Homo sapiens* (45.8% identity; 77.2% similarity), the catalytic subunit of *Pf*PKA displays distinctiveness in ATP anchoring and mechanisms of substrate recognition and phosphorylation. Selective targeting of these aspects would allow the tailoring of compounds that selectively inhibit *Pf*PKA with minimal cross inhibition of human PKA and consequent toxicity to the host.

Several studies using stimulants and inhibitors of PKA activity and modulation of gene expression have now highlighted the indispensability of *Pf*PKA for parasite growth.[11,12,14,15] A recent phospho-proteome study[16] identified 425 schizont phosphoproteins that display PKA phosphorylation motifs, indicating that this kinase likely has multiple important functions during the replicative phase of the asexual blood stage cell cycle. The role of *Pf*PKA is becoming increasingly well characterised in invasion of erythrocytes *via* phosphorylation of Apical Membrane Antigen 1 (*Pf*AMA1).[17] *Pf*AMA1 is a membrane spanning ligand with a pivotal role in erythrocyte invasion, namely formation of a 'moving junction'; a circumferential membrane junction that bridges the host–parasite interface and is thought to serve as a stable anchoring structure upon which the parasite can apply traction as it invades (reviewed in ref. 18). Within the parasite cytoplasm is the C-terminal domain of *Pf*AMA1, which is phosphorylated at serine S610 by *Pf*PKA. Mutation of $PfAMA1_{S610}$ renders parasites unable to efficiently invade and thus it appears that phosphorylation of $PfAMA1_{S610}$ by *Pf*PKA is a central event in establishing infection of erythrocytes.[17] Other substrates of *Pf*PKA have also been identified in recent times, namely myosin A (*Pf*MyoA), glideosome associated protein GAP45 (*Pf*GAP45) and calcium-dependent protein kinase 1 (*Pf*CDPK1), which all function during erythrocyte invasion.[16] Thus inhibition of *Pf*PKA will likely disrupt multiple key processes throughout erythrocyte invasion to halt the parasites' infection cycles.

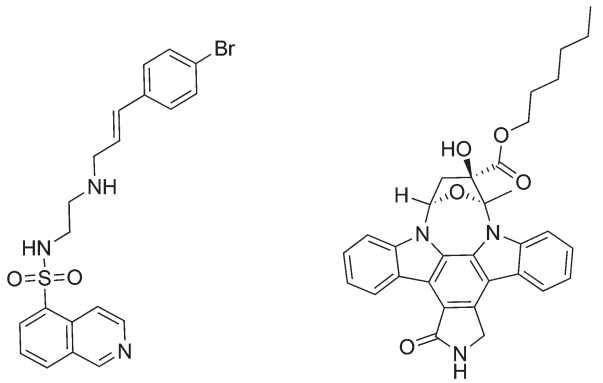


Fig. 1 Structures of commercially available PKA inhibitors H89 and KT5720.

Two commercially available PKA inhibitors, *N*-[2-[[3-(4-bromophenyl)-2-propenyl]amino]ethyl]-5-isoquinolinesulfonamide (H89)[19,20] and (5*R*,6*S*,8*S*)-hexyl 6-hydroxy-5-methyl-13-*oxo*-6,7,8,13,14,15-hexahydro-5*H*-16-*oxa*-4b,8a,14-triaza-5,8-methanodibenzo[*b*,*h*]cycloocta[*jkl*]cyclopenta[*e*]-as-indacene-6-carboxylate (KT5720)[21] (Fig. 1), have been used in over 2000 studies to investigate PKA activity, but widespread non-specific effects have been identified.[22–24] A series of 4-cyano-3-methylisoquinoline derivatives, **1–4** (Fig. 2), have also been reported as selective inhibitors of the catalytic subunit of PKA from rat liver, acting competitively with ATP as a substrate.[25] The unsubstituted compound, **2**, is commercially available and has been utilised as a chemical biology tool in a number of mammalian systems.[26,27] Preliminary study and testing of compound **4** and a small set of analogues against *P. falciparum* indicated sufficient activity to warrant further investigation.[56]

This report describes the preparation of a library of 4-cyano-3-methylisoquinoline analogues, based upon the structure of compound **4**, with a view to studying their structure–activity relationships as inhibitors of *Pf*PKA.

Results and discussion

Chemical synthesis

Synthesis of a library of 4-cyano-3-methylisoquinolines was undertaken principally through the key intermediate *N*-(4-cyano-3-methyl-1*H*-isochromen-1-ylidene)acetamide **6**, obtained by acetylation of commercially available 2-cyanophenylacetonitrile **5** (Scheme 1).[28]

To investigate the role of the alkyl substituent at the 3-position, **5** was reacted with two other anhydrides in the presence of sodium acetate. Residual amounts of the acid produced as a byproduct in such an acylation reaction can result in the rearrangement of the acylated intermediate into the corresponding isocoumarin.[29] As a consequence, analogues **7** and **8** were not isolated but were immediately coupled

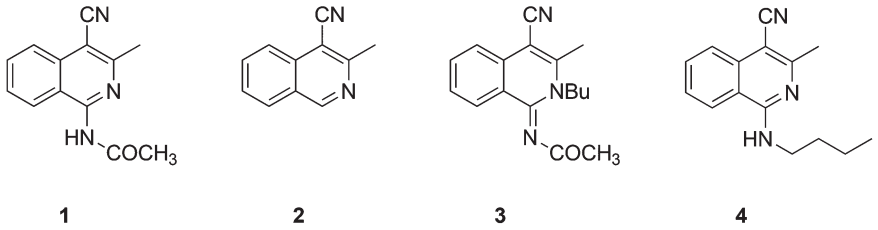
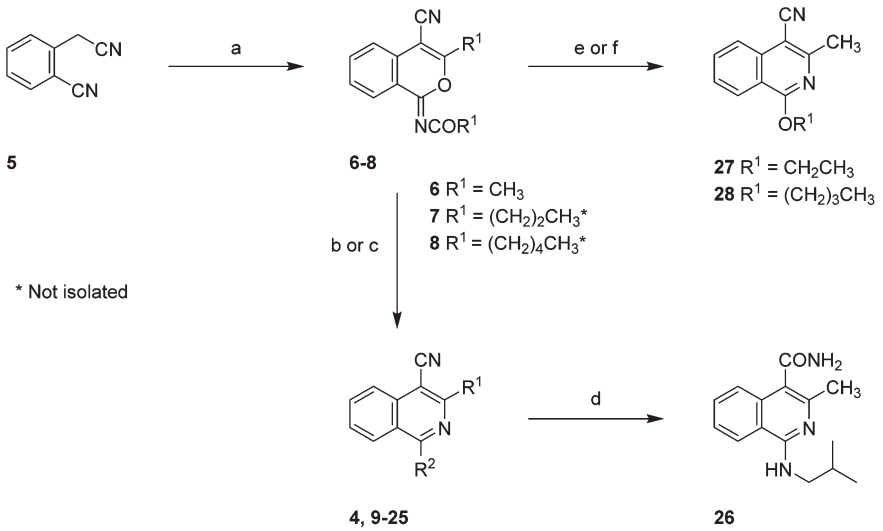


Fig. 2 Structures of known 4-cyano-3-methylisoquinoline inhibitors of rat liver PKA.



4 $R^1 = CH_3$, $R^2 = NH(CH_2)_3CH_3$
9 $R^1 = (CH_2)_2CH_3$, $R^2 = NH(CH_2)_3CH_3$
10 $R^1 = (CH_2)_4CH_3$, $R^2 = NH(CH_2)_3CH_3$
11 $R^1 = CH_3$, $R^2 = NH_2$
12 $R^1 = CH_3$, $R^2 = NH(CH_2)_5CH_3$
13 $R^1 = CH_3$, $R^2 = NH(CH_2)_7CH_3$
14 $R^1 = CH_3$, $R^2 = NHCH(CH_3)_2$
15 $R^1 = CH_3$, $R^2 = NHCH_2CH(CH_3)_2$
16 $R^1 = CH_3$, $R^2 = NHCH_2C(CH_3)_3$
17 $R^1 = CH_3$, $R^2 = NHCH_2CH(CH_3)CH_2CH_3$
18 $R^1 = CH_3$, $R^2 = NH(CH_2)_2CH(CH_3)_2$
19 $R^1 = CH_3$, $R^2 = NHCH(CH_3)CH_2CH(CH_3)_2$
20 $R^1 = CH_3$, $R^2 = NHCH(CH_3)(CH_2)_3N(CH_2CH_3)_2$
21 $R^1 = CH_3$, $R^2 = N(CH_2CH_2)_2O$
22 $R^1 = CH_3$, $R^2 = NH\text{-}CH(CH_3)Ph$ (*R*)
23 $R^1 = CH_3$, $R^2 = NH\text{-}CH(CH_3)Ph$ (*S*)
24 $R^1 = CH_3$, $R^2 = NHCH(Ph)_2$
25 $R^1 = CH_3$, $R^2 = NHCH(CH_2CH_3)_2$

Scheme 1 Synthesis of 1-, 3- and 4-substituted isoquinolines. Reagents and conditions: (a) $(R^1CO)_2O$, NaOAc, reflux, 2 h; (b) For **11**; aq. NH_3, 1,4-dioxane, reflux, 1 h;[29] (c) For **4**, **9–10** and **12–25**; R^2H, acetone, <25 °C, 0.5 h; (d) **15**, 1 : 1 H_2SO_4/H_2O, reflux, 65 h; (e) For **27**; KOEt, EtOH, reflux, 1 h; (f) For **28**; NaOBu, *n*-BuOH, reflux, 1 h.

with *n*-butylamine to produce the target compounds **9** and **10** with 3-propyl and 3-pentyl substituents respectively.

The reaction of acetamide **6** with amine nucleophiles has previously been described through a mechanism of ring opening and subsequent cyclisation to form the desired isoquinoline, including the lead compound **4**[30] and **11**.[29] Modification of the 1-position was explored through the synthesis of compounds **12–25** by treatment of the 3-methyl compound **6** in acetone at low temperature (<25 °C) with an extensive variety of linear and branched alkylamines, as well as aralkylamines.

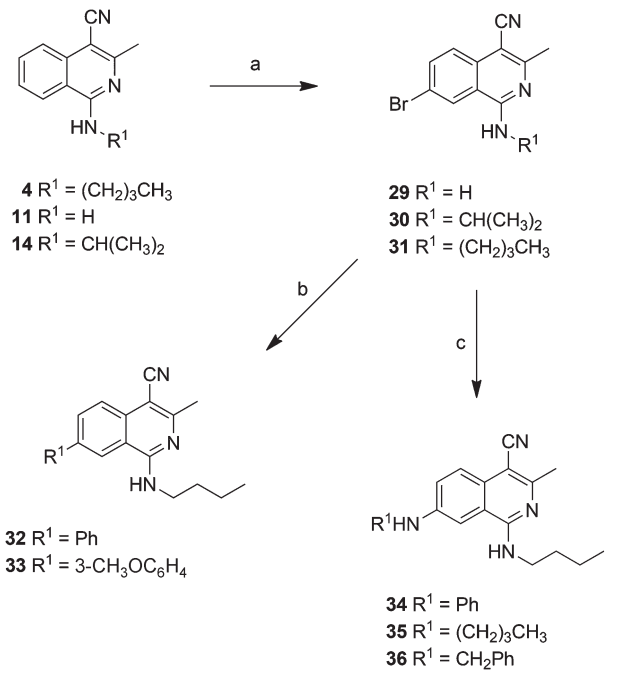
It was found that a large excess of amine was needed to force the complex mechanism to favor formation of the target isoquinolines. This resulted in some difficulties in the product isolation. For water soluble amines, addition of ice-water resulted in precipitation of the solid product. However, for the more hydrophobic alkylamines, such treatment resulted in an oily mix of the product and excess amine. Careful addition of dilute acid to the mixture was able to preferentially solubilise the amine so that the crude product could be separated. Chromatography or recrystallisation then completed the purification process. A number of variations on this theme were required for the various amines, and the details are given in the Experimental section.

Acid hydrolysis of **15** was carried out to afford the corresponding carboxamide **26**, to provide insight into the importance of the nitrile at the 4-position. Introduction of a 1-alkoxy moiety was also undertaken by the reaction of **6** with

ethoxide and butoxide in the corresponding anhydrous alcohols, to give the known 1-ethoxy compound **27**[29] and 1-butoxy compound **28**,[56] the latter providing a direct analogue of the lead compound **4**.

Electrophilic bromination of the benzo ring of compound **4** resulted in preferential formation of 7-bromo-1-butylamino-4-cyano-3-methylisoquinoline **31** (Scheme 2), with <10% of the 5-isomer. Carefully raising the pH to *ca.* 5 during aqueous workup resulted in predominant precipitation of the 7-isomer, and recrystallisation completed the purification. The orientation of the 7-bromo compound was established from NMR experiments. In particular, there was strong $^3J_{CH}$ coupling in the HMBC spectrum between C4 (at a characteristic δ 88.9 ppm) and H5, an *ortho*-coupled doublet at δ 7.63 ppm. This doublet therefore required the bromine to be at position 7. Similarly, $^3J_{CH}$ coupling was observed between C1 (δ 155.4 ppm) and H8, a *meta*-coupled doublet (δ 8.62 ppm). Brominated compounds **29** and **30** were also prepared without any further derivatisation.

Suzuki–Miyaura couplings of **31** with phenylboronic acid and *meta*-methoxyphenylboronic acid, in the presence of caesium fluoride and a catalytic amount of bis(diphenylphosphino)ferrocene]dichloropalladium(II) in a 4 : 1 mixture of acetonitrile and water, gave **32** and **33**, respectively. Buchwald–Hartwig amination of **31**, using potassium carbonate, tris(dibenzylideneacetone)dipalladium(0) and XPhos® in *tert*-butanol, with aniline, *n*-butylamine and benzylamine gave the corresponding products **34–36**.



4 $R^1 = (CH_2)_3CH_3$
11 $R^1 = H$
14 $R^1 = CH(CH_3)_2$

29 $R^1 = H$
30 $R^1 = CH(CH_3)_2$
31 $R^1 = (CH_2)_3CH_3$

32 R^1 = Ph
33 R^1 = 3-$CH_3OC_6H_4$

34 R^1 = Ph
35 $R^1 = (CH_2)_3CH_3$
36 $R^1 = CH_2Ph$

Scheme 2 Synthesis of 7-substituted isoquinolines *via* brominated analogue. Reagents and conditions: (a) H_2SO_4, NBS, 0 °C, 0.5 h; then 0 °C → rt, 2 h; (b) R^1-$B(OH)_2$, CsF, Pd(dppf)Cl_2, 4 : 1 CH_3CN/H_2O, 90 °C, 24 h; (c) R^1NH_2, K_2CO_3, $Pd_2(dba)_3$, XPhos®, *t*-BuOH, reflux, 17–18 h.

Derivatisation at the 7-position was further explored by nitration of the lead compound **4**, which, as for bromination, gave largely the 7-nitro isomer **37** (Scheme 3) and a trace amount of the 5-nitro isomer. Palladium-catalysed hydrogenation produced the desired amino analogue **38** in high yield. Reaction of **38** with acetyl chloride gave the acetamido compound **39**. Treatment of **38** with isocyanuric acid, produced *in situ* from sodium cyanate in 80% aqueous acetic acid, afforded the urea analogue **40**, along with a substantial amount of **39** as byproduct. Separation was readily achieved as only the latter was soluble in dichloromethane.

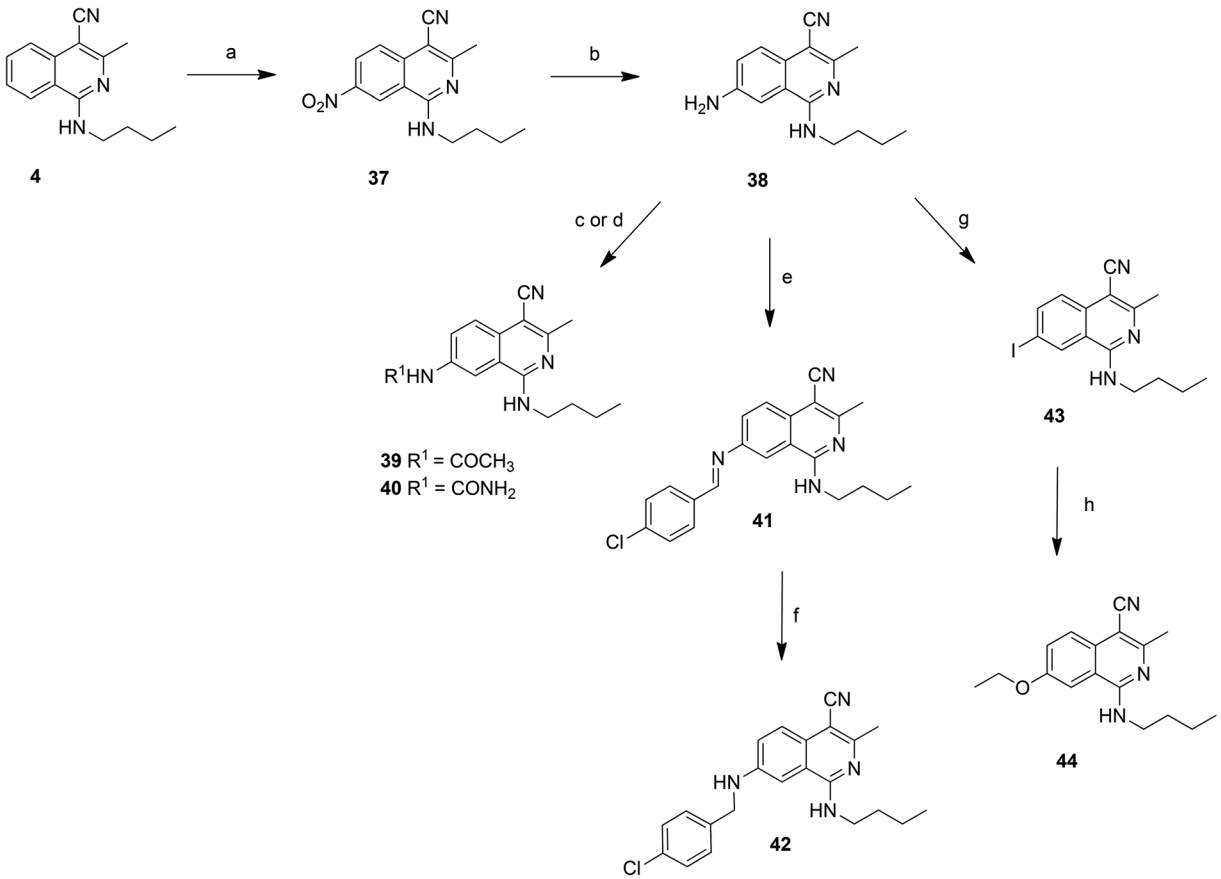
The 7-amino compound **38** was also reacted with 4-chlorobenzaldehyde in ethanol to generate the imine **41**, which was isolated and then reduced with sodium borohydride to the 7-(4-chlorobenzyl)amino compound **42** in a moderate yield. It was of interest to explore the activity of this substituent against *Pf*PKA as a series of 7-benzylamino-1-isoquinolinamine inhibitors had previously shown submicromolar inhibition against *P. falciparum*, with an unknown mechanism of action.[31] The amino group of **38** was also indirectly converted into an ether. Iodination of an intermediate diazonium salt gave the desired product **43**, which was reacted with ethanol in the presence of caesium carbonate, copper iodide and 1,10-phenanthroline to give the ethoxy ether **44**.[32]

Synthesis of analogues exploring the 5-position of the isoquinoline exploited the previously prepared 7-bromo isomer **31**. Nitration of **31** gave the 7-bromo-5-nitro compound **45** (Scheme 4). Suzuki–Miyaura coupling, as for **31**, gave the 5-nitro-7-phenyl analogue **46**. Palladium-catalysed hydrogenation of **45** in the presence of triethylamine reduced the nitro group and removed the bromine to afford the 5-amino compound **47** in high yield. Acetylation, as before, gave **48**. Imine **49** was generated by the reaction of 4-chlorobenzaldehyde with **47**, following methodology used for the 7-isomer **41** but, in this instance, reflux was required. This sluggishness was most likely due to the steric hindrance by the nitrile at the *peri* 4-position. Reduction of imine **49** by sodium borohydride afforded the 5-(4-chlorobenzyl)amino compound **50**.

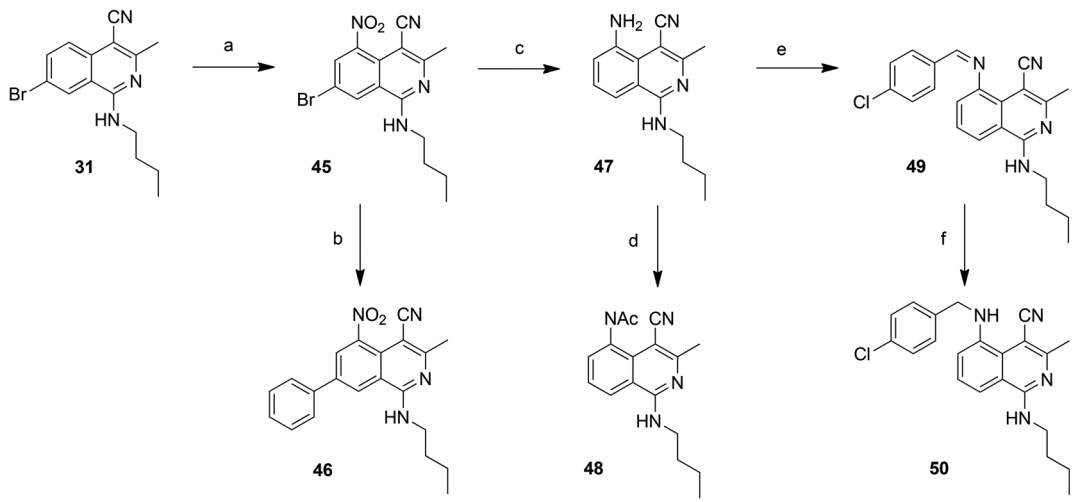
Inhibitory potency of 4-cyano-3-methylisoquinolines against parasite growth

Biological testing of the compounds was carried out on asexual blood stages of *Plasmodium falciparum* chloroquine sensitive 3D7 and resistant W2mef strains. W2mef parasites possess mutations in the chloroquine transporter, which confer greater resistance to the antimalarial, and an extra copy of the multi-drug resistance transporter, which boosts efflux of antimalarial drugs.[33–35] Testing was carried out using a Malstat assay to measure parasite lactate dehydrogenase levels, which correlate with parasite proliferation.[36] Assay results from the isoquinolines with modifications at the 1-, 3-, and 4-positions are shown in Table 1.

It was observed that extension of the 3-methyl group of **4** resulted in a moderate improvement in potency of both **9** and **10**, with the 3-propyl and 3-pentyl substituents respectively.

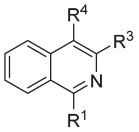


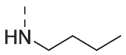
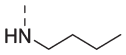
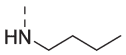
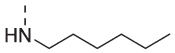
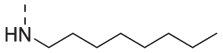
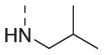
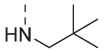
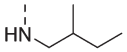
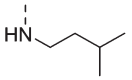
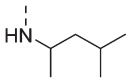
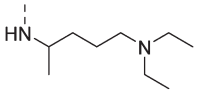
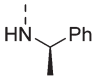
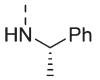
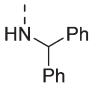
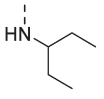
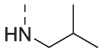
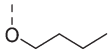
Scheme 3 Synthesis of 7-substituted isoquinolines *via* nitrated analogue. Reagents and conditions: (a) H_2SO_4, KNO_3, 0 °C, 0.5 h; (b) 10% Pd/C (8% w/w), H_2, EtOH, rt, 0.5 h; (c) For **39**; AcCl, Et_3N, THF, 0 °C, 5 min; then 0 °C → rt, 2 h; (d) For **40**; NaOCN, 5 : 1 AcOH/H_2O, rt, 16 h; (e) 4-ClC_6H_4CHO, EtOH, rt, 2 h; (f) $NaBH_4$, EtOH, rt, 16 h; (g) $NaNO_2$, concd. HCl, −5 °C, 1 h; KI, H_2O, −5 °C → rt, 4 h; (h) CuI, 1,10-phenanthroline, $CsCO_3$, EtOH, 110 °C, 18 h.



Scheme 4 Synthesis of 5-substituted isoquinolines. Reagents and conditions: (a) H_2SO_4, KNO_3, 0 °C, 1 h; (b) $PhB(OH)_2$, CsF, $Pd(dppf)Cl_2$, 4 : 1 CH_3CN/H_2O, 90 °C, 24 h; (c) 10% Pd/C (8% w/w), H_2, Et_3N, EtOH, rt, 2 h; (d) AcCl, Et_3N, THF, 0 °C, 5 min; then 0 °C → rt, 4 h; (e) 4-ClC_6H_4CHO, EtOH, reflux, 24 h; (f) $NaBH_4$, EtOH, rt, 16 h.

Table 1 Inhibition of malarial parasite 3D7 and W2mef proliferation by 1-, 3- and 4-substituted isoquinoline analogues[a]



	R^1	R^3	R^4	3D7 IC_{50} (μM) ± SD	W2mef IC_{50} (μM) ± SD
4		CH_3	CN	34 ± 10	33 ± 8.7
9		$(CH_2)_2CH_3$	CN	13 ± 3.6	11 ± 1.1
10		$(CH_2)_4CH_3$	CN	6.0 ± 0.3	6.7 ± 0.4
11		CH_3	CN	34 ± 12	36 ± 10
12		CH_3	CN	5.7 ± 2.6	2.4 ± 2.6
13		CH_3	CN	16 (n = 1)	8.8 (n = 1)
14		CH_3	CN	27 ± 12	>50
15		CH_3	CN	>50	28 ± 5
16		CH_3	CN	>50	>50
17		CH_3	CN	14 ± 4.3	17 ± 2.7
18		CH_3	CN	10 ± 5.7	14 ± 8.0
19		CH_3	CN	2.7 ± 0.8	2.5 ± 1.2
20		CH_3	CN	15 ± 0.7	2.8 ± 2.3
21		CH_3	CN	40 ± 17	>50
22		CH_3	CN	9.7 ± 3.3	6.7 ± 1.4
23		CH_3	CN	0.6 ± 0.4	1.0 ± 0.6
24		CH_3	CN	10 ± 3.1	9.4 ± 1.4
25		CH_3	CN	1.0 ± 0.5	0.7 ± 0.8
26		CH_3	$CONH_2$	>50	32 ± 4.2
27		CH_3	CN	>50	>50
28		CH_3	CN	36 ± 4.0	16 ± 0.1

[a] Data represents the mean ± SD for two or three independent experiments unless otherwise specified.

Removal of the alkyl group to amino compound **11** retained similar activity comparable to the lead compound **4**. Extending the length of the 1-alkylamino chain was found to be favorable, with a lower IC_{50} observed for the 1-hexylamino analogue **12**. This effect was reduced for the largest 1-octyl extension of **13** against 3D7 parasites, but not for the W2mef parasites.

Branching of the 1-alkyl substituents was also explored, with a mixture of structure–activity results. At best, inhibition similar to the lead compound **4** was observed for compounds **14**, **15** and **16** or no inhibition at all, depending on the parasite tested. The branched butylamino groups of **17** and **18**, in comparison with lead compound **4**, gave only a modest improvement of inhibition over the lead compound **4** but the (±)-1-((4-methyl-2-pentyl)amino) analogue **19** obtained an order of magnitude improvement in potency with an IC_{50} of 2.7 ± 0.8 μM against 3D7 parasites and 2.5 ± 1.2 μM against W2mef parasites. It was noted that **17**, **19** and **20** contained a chiral stereocentre, however the inhibitory activity of these compound was not sufficient to warrant separation and testing of each of the enantiomers. The (±)-1-((5-diethylamino)2-pentyl)amino) substituent of the known malarial treatment chloroquine gave compound **20**, which was found to be a better inhibitor of W2mef than 3D7 parasites.

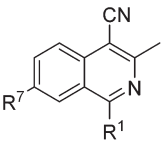
The introduction of the 1-morpholino moiety for compound **21** was found to reduce growth inhibitory activity. However, we were delighted to observe that alpha-methylation of the 1-benzylamino functional group resulted a significant improvement in parasite growth inhibition, in particular the *S*-enantiomer **23** with an IC_{50} of 0.6 ± 0.4 μM against 3D7 parasites and 1.0 ± 0.6 μM against W2mef parasites. This result, together with the potency of **19** and, to a lesser extent, **20**, suggested that inhibitory activity was favoured by to the presence of an alpha-methyl group. Attempts to replicate this low micromolar potency without requiring a stereocentre were made, with some inhibition observed for the biphenyl compound **24** but, very pleasingly, the achiral 1-ethylpropylamino analogue **25** was found to have an IC_{50} of 1.0 ± 0.5 μM against 3D7 parasites and 0.7 ± 0.8 μM against W2mef parasites. The large standard deviations observed for some compounds resulted from the fact that the IC_{50} modestly differed between assay repeats (n = 2 or 3 unless otherwise stated). This could be due the compounds acting during narrow replicative phase of the 48 h cell cycle which the parasites would be expected to pass through once or twice during the 72 h assay period. Parasites that attempted to pass though replication twice could be more adversely affected than those that passed through once. Although reasonable attempts were made to synchronise the parasites similarly between repeats so that they would only replicate once, in some repeats there might have been a greater proportion that attempted to replicate twice.

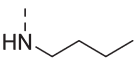
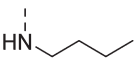
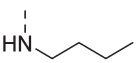
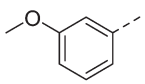
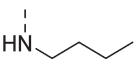
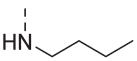
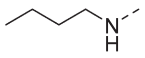
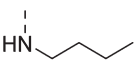
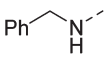
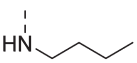
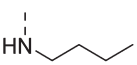
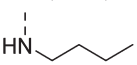
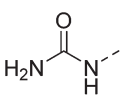
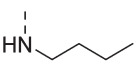
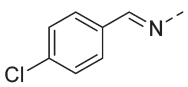
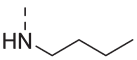
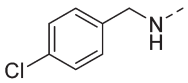
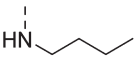
The importance of the 1-alkylamino functionality to the activity of this family of compounds was also demonstrated by the substitution of a 1-alkoxy functional group, with modest to poor inhibition observed for the 1-butoxy analogue **28** when compared the lead compound **4** and the 1-ethoxy analogue **27** not producing any inhibition of the assay at 50 μM. Reduction of the 4-nitrile functional group of **15** to the corresponding amide of **26** did not result in any improvement of inhibitory activity against the parasites.

The results of the testing of the 7-substituted isoquinoline analogues in the Malstat assay are presented in Table 2. Bromination at the 7-position usually resulted in moderate inhibitors of parasite growth as observed for analogues **29** and **31** but not for compound **30**.

Introduction of an unsubstituted phenyl ring by Suzuki–Miyaura coupling was found to be detrimental to the activity of

Table 2 Inhibition of malarial parasite 3D7 and W2mef proliferation by 7-substituted isoquinoline analogues[a]

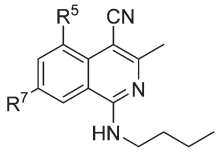


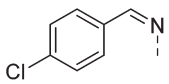
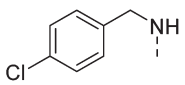
	R^1	R^7	3D7 IC_{50} (μM) ± SD	W2mef IC_{50} (μM) ± SD
29			15 ± 2.3	11 ± 2.8
30			>50	>50
31			12 ± 4.8	2.5 ± 2.1
32			>50	>50
33			5.7 ± 0.1	15 ± 0.2
34			5.9 ± 1.5	5.0 ± 1.2
35			18 ± 2.3	24 ± 6.8
36			18 ± 3.3	21 ± 2.0
37			5.5 ± 0.7	4.3 ± 1.8
38			31 ± 6.9	15 ± 3.3
40			9.7 ± 13	6.1 ± 1.4
41			27 ± 3.8	>50
42			5.4 ± 3.6	5.2 ± 2.0
44			29 ± 12	16 ± 6.7

[a] Data represents the mean ± SD for two or three independent experiments unless otherwise specified.

the compound **32**, but the *meta*-methoxyphenyl substituent of **33** and the extended 7-anilino compound **34** showed good inhibitory activity. Neither the 7-butylamino or 7-benzylamino compounds, **35** and **36** respectively, were particularly potent. However, the addition of a *p*-chloro substituent to the benzylamine functional group resulted in the *p*-chlorobenzylamino compound **42** which was observed to lead to a good improvement in activity, which was not observed for the corresponding imine precursor **41**, suggesting the potential for a hydrogen bonding interaction.

Table 3 Inhibition of malarial parasite 3D7 and W2mef proliferation by 5-substituted isoquinoline analogues[a]



	R^7	R^5	3D7 IC_{50} (μM) ± SD	W2mef IC_{50} (μM) ± SD
45	Br	NO_2	0.7 ± 0.2	0.6 ± 0.4
46	Ph	NO_2	13 ± 8.8	4.4 ± 1.9
47	H	NH_2	13 ± 1.8	23 ± 14
48	H		>50	>50
49	H		9.2 ± 4.1	4.2 ± 1.7
50	H		1.2 (n = 1)	1.8 (n = 1)

[a] Data represents the mean ± SD for two or three independent experiments unless otherwise specified.

While 7-nitration resulted in compound **37**, which was a good inhibitor of parasite growth, reduction to the 7-amino analogue returned compound **38** which had only similar potency to that of the lead compound **4**. Conversion of the 7-amino functional group to the 7-urea substituent of **40** resulted in a moderately potent analogue. The 7-ethoxy analogue **44** was found to be comparable to the lead compound **4** against the parasites, with some improvement in inhibitory activity against the W2mef strain.

Finally, results from the Malstat assays conducted using the 5-substituted analogues synthesised are shown in Table 3.

Nitration of the 7-bromo analogue **31** resulted in compound **45** which was found to be equipotent with inhibitors **23** and **25** with an IC_{50} of 0.7 ± 0.2 against 3D7 parasites and 0.6 ± 0.4 against W2mef parasites. Suzuki coupling of **45** with phenylboronic acid gave analogue **46**, which was observed to have only moderate potency. Reduction of the 5-nitro compound **45** to the 5-amino compound **47** improved potency only modestly compared to **4**, which again was completely removed by acetylation to analogue **48**. An improvement of inhibition was observed for both imine compound **49** and the *p*-chlorobenzylamino analogue **50**, compared to 7-substituted analogues of **41** and **42**.

Overall, it was observed that an increase in the 1-aminoalkyl chain length or the introduction of a bulkier substituent generally resulted in an increase in compound potency. The presence of alpha-branching on the alkyl chain was determined to be the most crucial structural change resulting in a much greater increase in activity over the lead compound **4**. Substitution of the 1-amino moiety by 1-ethoxy or the morpholino functional group removed all activity. Extension of the 3-methyl group was also found to be acceptable, while hydrolysis of the nitrile substituent was not tolerated. Analogues were generally better tolerated at the 5-position over the 7-position of the compound, with most changes increasing potency moderately. A summary of the structure–activity relationships is shown in Fig. 3.

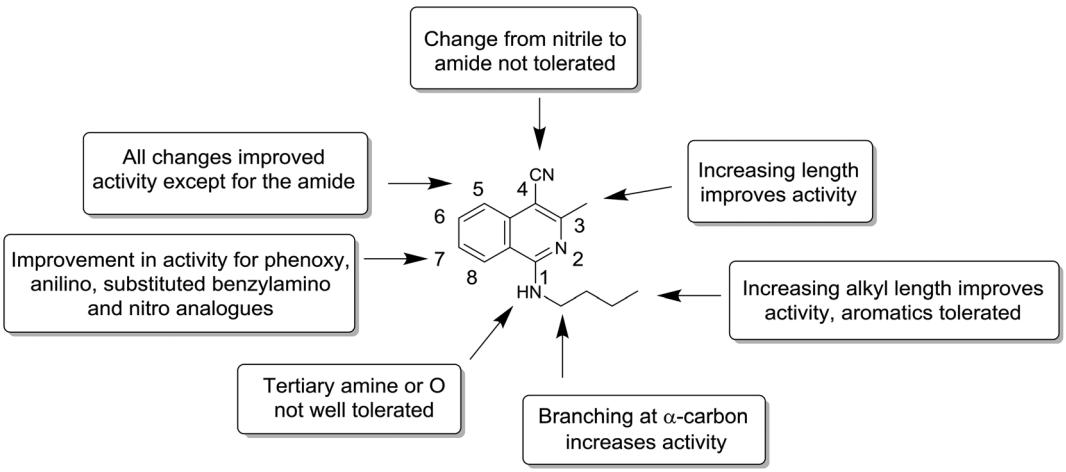


Fig. 3 Structure–activity relationships summary of analogues of 4-cyano-3-methylisoquinoline **4**.

Compounds **23** and **25** were among the most potent growth inhibitors tested and represented a significant improvement over the lead compound **4**. Both are drug-like compounds which comply with Lipinki's rule of 5, which predicts poor absorption or permeation is more likely with molecular weights of more than 500 Daltons, more than 5 hydrogen bond donors, more than 10 hydrogen bond acceptors and a calculated log *P* of greater than 5.[37] Good oral bioavailability is also predicted for these compounds which all possess less than 10 rotatable bonds and total polar surface area (tPSA) of less than 140 Å^2.[38] These compounds, like most that were tested here, had similar levels of growth inhibition against 3D7 and W2mef *P. falciparum* strains, indicating that neither of the chloroquine resistance mechanisms increased the tolerance for the compounds, boding well for potential therapeutic use.

Before progressing to *Pf*PKA inhibition assays we ensured that the most potent compounds were not inhibiting the Malstat reaction and thereby giving the false impression they were reducing parasites growth. A counter screen was performed on fresh 3D7 parasite culture to which **4**, **12**, **13**, **23** and **25** had been added to 100 μM. Relative to a no drug control, the compounds only weakly inhibited the screen reaction by 5–10%, much less than the ~100% inhibition evident in the growth assays (ESI†). The weak counter screen inhibition was probably due to the DMSO carrier.

Specificity of 4-cyano-3-methylisoquinolines for *Pf*PKA

An ELISA-based assay was developed to measure *Pf*PKA activity *via* phosphorylation of a recombinant version of the 56 amino acid, C-terminal cytoplasmic tail of *Pf*AMA1 expressed as a fusion protein with glutathione S transferase (GST) (Fig. 4A). The GST-AMA1 fusion proteins were used to coat the wells of ELISA plates for subsequent phosphorylation. To obtain a source of *Pf*PKA we attempted to express a His-6 tagged version of the catalytic domain of *Pf*PKA that was codon optimised for expression in *Escherichia coli* but the protein was insoluble and therefore likely inactive. Instead we used lysates of late stage intraerythrocytic *P. falciparum* that contained active *Pf*PKA. Because the lysate also contained other active kinases, we used a panel of mutant *Pf*AMA1 C-terminal fusion proteins to derive specific *Pf*PKA activity. In addition to the wildtype AMA1 tail, a *Pf*AMA1$_{S610only}$ tail was utilised where S610 is the *Pf*PKA target site[17] and all other phosphorylatable residues are mutated, allowing only phosphorylation by *Pf*PKA on this substrate. The inverse of this tail was also generated where only S610 is mutated allowing this substrate to accept phosphorylation from multiple kinases except for *Pf*PKA (Fig. 4A). A commercial phospho-serine antibody was used to detect any serine phosphorylation of the AMA1 tail as well as an antibody raised against *Pf*AMA1 tail peptide phosphorylated at S610 to specifically detect only *Pf*PKA-specific (S610) phosphorylation (Fig. 4B). To validate this assay as being quantitative for *Pf*PKA activity, the tail substrates were also used with or without added cAMP and the PKA inhibitor H89. As expected, S610 phosphorylation was maximally detected on only the *Pf*AMA1$_{WT}$ and *Pf*AMA1$_{S610only}$ substrates from lysates supplemented with cAMP while H89 strongly inhibited cAMP-dependent phosphorylation (Fig. 4B). This indicates that the ELISA method incorporating parasite lysates supplemented with cAMP could be used to quantitatively and specifically measure *Pf*PKA activity with the specific AMA1 tail phospho-S610 antibody achieving a great signal to noise ratio.

Next we attempted to determine the effect of the most growth inhibitory 4-cyano-3-methylisoquinolines on *Pf*PKA activity. Compounds **4**, **12**, **13**, **23**, **25** and **45** were titrated into the ELISA-based phosphorylation assays along with H89 as a positive control. While H89 clearly inhibited phosphorylation of *Pf*AMA1 at S610, none of the 4-cyano-3-methylisoquinolines had a significant effect on the phosphorylation levels even at concentrations as high as 100 μM, indicating that they do not target *Pf*PKA (Fig. 5).

Invasion inhibitory capacity of 4-cyano-3-methylisoquinolines

The lack of inhibition by the 4-cyano-3-methylisoquinoline compounds on *Pf*PKA was surprising given that the original compound **4** was found to be active against rat PKA.[26] Given that the 4-cyano-3-methylisoquinolines are thought to competitively inhibit ATP binding, we hypothesised that these compounds might target an enzyme with an ATP binding pocket. Many signaling enzymes function late in the cell cycle as they are utilised extensively to coordinate egress and invasion, therefore the effect of these compounds upon erythrocyte egress and invasion was examined. Assays to quantify egress and invasion inhibition were performed using transgenic parasites that express and secrete nanoluciferase (NanoLuc) into the parasitophorous vacuole (PV).[39,40] The most growth inhibitory compounds, **4**, **12**, **13**, **23**, **25** and **45**, were added to pre-invasion cultures and incubated for 4 hours over which time the parasites attempted to egress and invade new erythrocytes. After the 4 hour incubation period, parasite media containing NanoLuc released from the ruptured host cell and PV was harvested for analysis of egress inhibition. To quantify invasion inhibition, the 4-cyano-3-methylisoquinolines were washed out after the 4 hour invasion window and the cultures were treated to prevent further invasions, thereby only including parasites that invaded during drug treatment. These parasites were allowed to grow for a further 24 hours until NanoLuc expression was high enough to measure. To quantify the levels of NanoLuc in the egress media or in the invaded parasite cultures, NanoGlo substrate was added and relative light units (RLU) were measured. The RLUs were normalised against those for egress or invasion in untreated cultures (100%) and in uninfected cultures (0%) and inhibition curves were plotted. All of the compounds poorly inhibited egress except at the highest concentrations (Fig. 6). In comparison, the compounds were much more potent at inhibiting invasion with compound **45** being the most effective. This indicates that the target of the 4-cyano-3-methylisoquinolines probably functions during the invasion stage of the life cycle and may

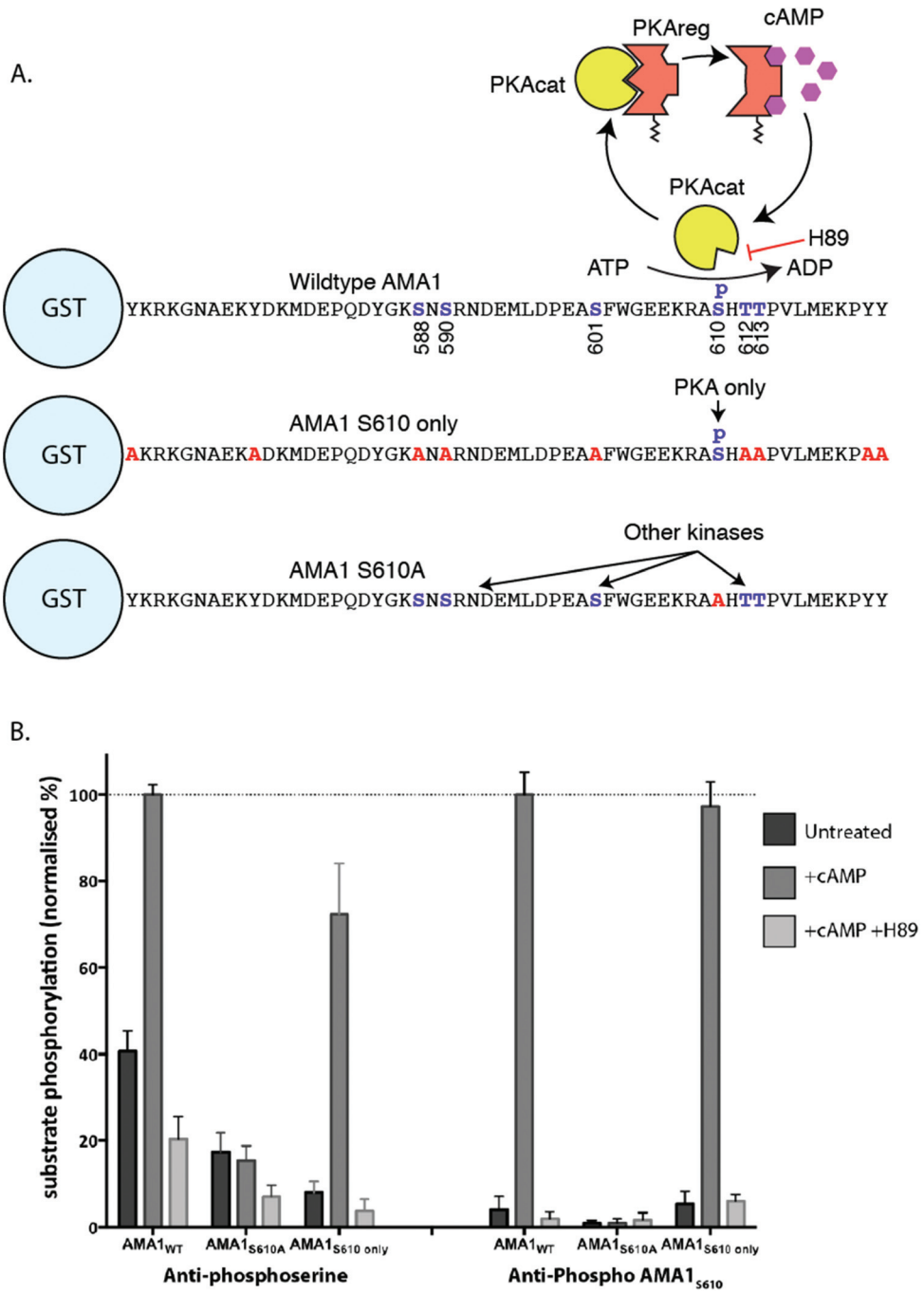


Fig. 4 An ELISA-based method can be used to measure *Pf*PKA activity against the S610 residue of *Pf*AMA1 expressed as a GST-AMA1 tail fusion protein. A) Diagram of a panel of GST-AMA1 tail fusion proteins showing how they were modified by *Pf*PKA and other kinases. B) Immobilised recombinant *Pf*AMA1$_{WT}$, *Pf*AMA1$_{S610A}$ and *Pf*AMA1$_{S610only}$ fusion proteins were phosphorylated with parasite lysates supplemented with cAMP and H89 to trigger and inhibit *Pf*PKA activity, respectively. Following the reactions, an ELISA was performed to detect phosphorylation of S610 (the *Pf*PKA site) using a commercial phosphoserine antibody and a specific antibody against a *Pf*AMA1 peptide phosphorylated at S610. As expected, maximal *Pf*PKA-specific signals resulted from phosphorylation of the *Pf*AMA1$_{WT}$ and *Pf*AMA1$_{S610only}$ substrates by parasite lysates supplemented with cAMP.

be another kinase(s) that phosphorylates invasion proteins similar to *Pf*AMA1.

To more precisely observe the phenotype of parasites treated with the 4-cyano-3-methylisoquinolines, live cell video microscopy was performed on late-stage W2mef parasites as they egressed and invaded new erythrocytes. The basic steps of egress and invasion are as follows. Before rupture and release of daughter parasites, the infected erythrocyte which is

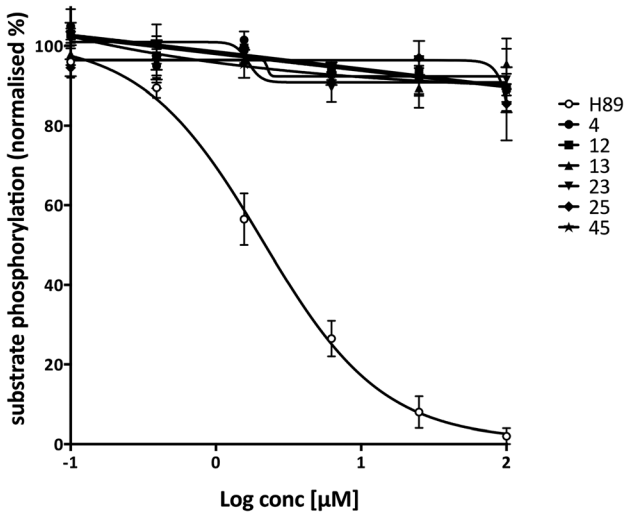


Fig. 5 Effect of the 4-cyano-3-methylisoquinolines on *Pf*PKA activity. The compounds were titrated into the ELISA-based phosphorylation assay for detection of *Pf*PKA activity *via* *Pf*AMA1$_{S610only}$ phosphorylation but were found to have no effect at their growth inhibitory concentrations, indicating that they do not target *Pf*PKA.

normally flattened rounds up into a sphere and the hemozoin becomes highly condensed.[41] Egress begins with the sudden ejection of a few merozoites from a pore in the erythrocyte surface followed by curling back of the membranous edges of the pore further opening up the erythrocyte and ejecting more merozoites.[42] Erythrocyte invasion is a three step process beginning with the pre-invasion phase where the merozoite contacts and deforms the erythrocyte surface.[43,44] This phase lasts about 10 seconds during which the merozoite reorientates its penetrative apical end onto the erythrocyte surface. Then the active invasion phase lasts about 10 seconds during which a vacuole forms around the invading merozoite.[44] During the final post-invasion phase, the internalised merozoite and erythrocyte become separated when scission of the membranous isthmus connecting the vacuole and erythrocyte membranes occurs. Precisely when this happens is difficult to visualise because 30 seconds after invasion the erythrocyte surface becomes spiculated and the merozoite inside is difficult to see.[44] Over several minutes the erythrocyte recovers its normal biconcave shape by which time the merozoite has differentiated into an amoeboid ring. Due to the amount of effort and compound required for this method, achiral compound **25** was selected for testing at a concentration of 10 μM, which conferred about 70% invasion inhibition in the NanoLuc assay. Of 14 schizonts treated with **25** that ruptured, eight appeared normal with each liberating 12–24 merozoites. Six ruptures were aberrant and released a single incompletely divided schizont (Fig. 7). From the eight normal ruptures, 30 merozoites were observed to contact and deform their surrounding erythrocytes and of these 15 invaded normally and progressed to form intra-erythrocytic ring-stage parasites

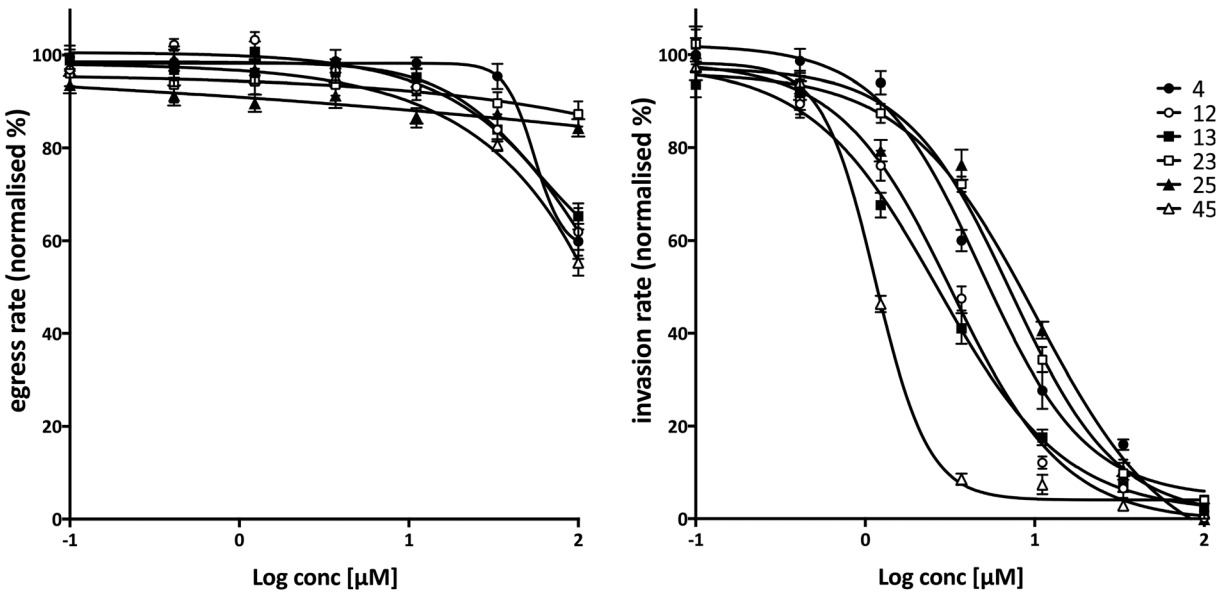


Fig. 6 Inhibition of parasite egress and invasion by 4-cyano-3-methylisoquinolines. The most growth inhibitory compounds were tested for their egress and invasion inhibition capacity to determine whether this stage of the life cycle is affected and when the drug target(s) may function. The compounds were titrated into NanoLuc-expressing late stage 3D7 cultures for 4 hours covering a period of egress and invasion. The levels of NanoLuc released into the media during this period were a marker for parasite egress and the dose–response curves indicate the compounds did not substantially inhibit egress except at higher concentrations. To measure invasion inhibition the compounds were removed after 4 hours and the parasites were grown for 24 hours to increase the NanoLuc signal in the parasites. Lysis of the whole cultures and measurement of NanoLuc levels indicated that the compounds were much more effective against invasion than egress. The data has been normalised against untreated cultures (100%) and uninfected cultures (0%). Assays were conducted in triplicate and error bars denote one standard deviation.

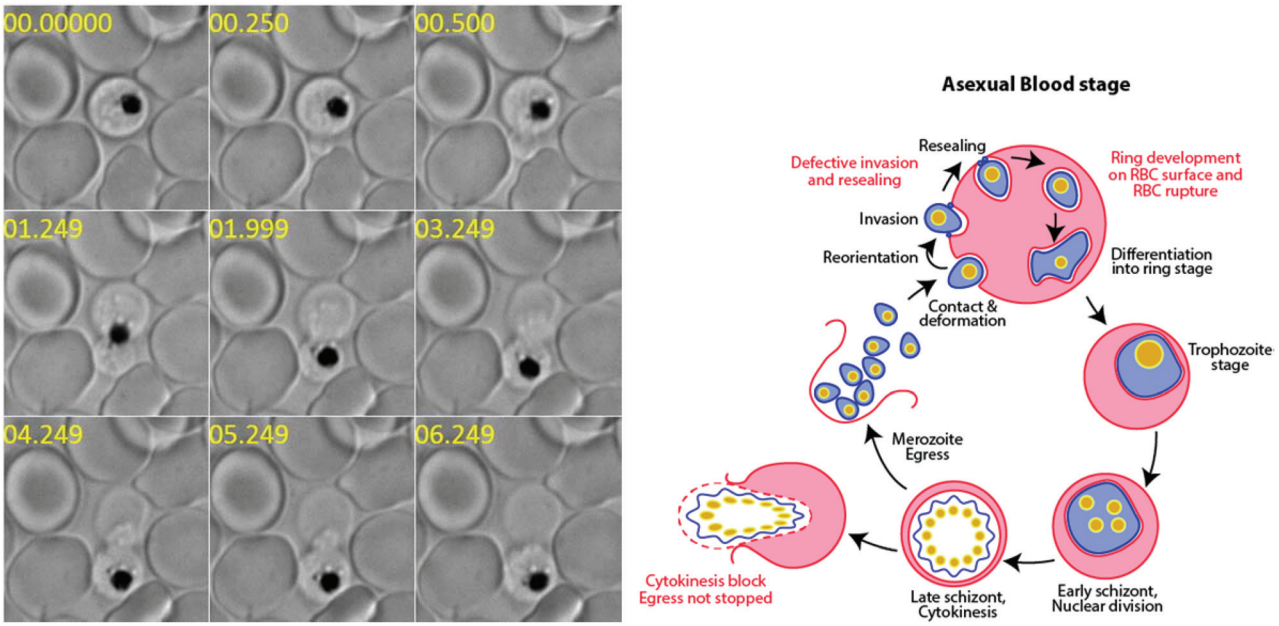


Fig. 7 Live cell microscopy panels and a diagram of invasion in the presence of compound **25**. At 10 μM, **25** blocked cytokinesis as indicated in the live cell micrograph (time points in seconds are shown). Compound **25** blocked multiple points of the invasion phase after cytokinesis including active invasion and resealing of the host cell.

(Videos S1 and S2,† white arrows). The remaining merozoites however failed to form viable ring-stage parasites and exhibited three general kinds of abnormal behavior (Fig. 7). The most upstream blockage occurred in about half the unsuccessful merozoites where they deformed their erythrocyte but did not cleanly invade and instead seemed to embed themselves into the erythrocyte surface (Videos S1 and S2†). This triggered echinocytosis of the erythrocyte and as it recovered its normal shape several minutes later the merozoites or by now differentiated ring-stage parasites, remained attached to the outside (Videos S1 and S2†). Other aberrant merozoites appeared to enter the erythrocytes normally but later appeared to exit at the invasion point either as a merozoite or ring. Some invading merozoites remained internal and formed rings but soon triggered lysis of their host erythrocyte. This indicates that the target of compound **25** has a role in cytokinesis, invasion and resealing. The observed defects suggests that active invasion powered by the actomyosin invasion motor is not functioning correctly. Proteins within this motor such as GAP45 are known to be phosphorylated by kinases such as PKA, PKB, PKG and CDPK1.[16] A notable surprise from the live cell imaging was the egress of whole undivided schizonts. It seems unlikely that these were immature schizonts that had not yet formed merozoites and had been stimulated to prematurely egress because there was no increase in egress following the addition of compound **25** according to the NanoLuc assay. It is possible that when compound **25** was added to schizonts before they had undergone cytokinesis, the process was arrested but the egress program continued unimpeded. If cytokinesis was complete or nearly so when compound **25** was added, merozoites were released but were unable to invade properly. Known phosphorylation targets involved in cytokinesis and formation of the inner membrane complex such as Rab11A could be functionally compromised.[16,45]

Screening for potential kinase targets of 4-cyano-3-methylisoquinoline inhibitors

Given that the lead compound **4** is thought to have competitive ATP-binding activity against rat PKA, we reasoned that the synthesised compounds might exhibit a similar mode of action albeit against a different parasite kinase. To identify potential kinase targets of the compounds, we introduced compound **25** into a commercial human kinase screen (MRC PPU International Centre for Kinase Profiling, University of Dundee, United Kingdom) and tested a panel of available kinases with homologs in *P. falciparum*, where inhibited human kinases might indicate a parasite target given the homology. Aurora B kinase, glycogen synthase kinase 3-beta, cyclin-dependent kinase 2, cyclin-dependent kinase 9 and cyclin-dependent kinase like were found to have reduced activity in the presence of 10 μM **25** (29%, 38%, 32%, 54% and 53% respectively) (Table 4). In support of our *Pf*PKA assays was the observation that **25** did not inhibit Human PKA indicating that modification of **4** to **25** had ablated activity against mammalian PKA.

These kinases (*Pf*ARK2, *Pf*GSK3, *Pf*PK5, *Pf*CLK1 and *Pf*CRK1) in *P. falciparum* are known to have roles in mitosis, cell cycle regulation and transcription, among others.[46–48,57] Specifically, aurora kinase B, which was inhibited to the greatest extent, is required for chromosome condensation and segregation as well as for cyto-

Table 4 Inhibition of human kinases[a]

Hs kinase tested	*Pf* Kinase homolog	Activity after treatment (%)	Function
PKA (AGC kinase)	PKA (PFI1685w)	92 ± 7	
PKB (AGC kinase)	PKB (PFL2250c)	122 ± 22	
PKC (AGC-related kinase)	PKC (PF11_0464)	96 ± 24	
PDK1 (AGC-related kinase)	Putative (PF11_0227)	78 ± 31	
Aurora A (aurora kinase)	ARK1 (PFF0260w)	87 ± 5	
Aurora B (aurora kinase)	ARK2 (PFC0385c)	29 ± 2	Mitosis
GSK3β (glycogen synthase kinase)	GSK3 (PFC0525c)	38 ± 6	Multiple
CK1 (casein kinase)	CK1 (PF11_0377)	117 ± 16	
CK2 (casein kinase)	CK2 (PF11_0096)	87 ± 1	
CDK2-Cyclin A (cyclin-dependent kinase)	PK5 (MAL13P1.279)	32 ± 3	Cell cycle
CDK9-Cyclin T1 (cyclin-dependent kinase)	CRK1 (PFD0865c)	54 ± 5	Cell cycle
CLK2 (cyclin-dependent kinase like)	CLK1 (PF14_0431)	53 ± 2	Transcription
ERK1 (mitogen-activated protein knase)	MAP2 (PF11_0147)	133 ± 11	
CAMK1 (calcium/calmodulin-dependent kinase)	PK2 (PFL1885c)	98 ± 1	
NEK2a (NIMA kinase)	NEK1 (PFL1370)	84 ± 19	
EIF2aK3 (eukaryotic translation initiation factor 2a kinase)	PK4 (PFF1370w)	96 ± 1	
MEKK1 (tyrosine kinase like)	TLK1 (PFB0520w)	99 ± 4	

[a] Data represents the mean % activity remaining of assay duplicates ± SD.

kinesis in eukaryotes. *Pf*ARK2 being a target of the 4-cyano-3-methylisoquinoline inhibitors may explain the abnormal phenotype observed where parasites do not complete division before egressing in the presence of **25**.[49] In future studies, these compounds will be tested against recombinant forms of these parasite-specific kinases to elucidate the target of the inhibitors.

Conclusion

The large evolutionary distance between mammals and *Plasmodium* and the subsequent divergence in sequence identity between orthologous enzymes such as kinases, makes it feasible to repurpose mammalian enzyme inhibitors towards greater activity against their *Plasmodium* orthologs at little expense to the original mammalian enzyme. For this reason, the synthesis and structure–activity relationships of a library of synthesised 4-cyano-3-methylisoquinolines and related analogues have been explored. Low micromolar inhibition of parasite growth was observed against both 3D7 and chloroquine-resistant W2mef strains of *Plasmodium falciparum* for compounds **23** (IC_{50} 0.6 ± 0.4 μM against 3D7 parasites and IC_{50} 1.0 ± 0.6 μM against W2mef parasites) and **25** (IC_{50} 1.0 ± 0.5 μM against 3D7 parasites and IC_{50} 0.7 ± 0.8 μM against W2mef parasites) but, surprisingly, the compounds appeared to have no effect on their supposed target of *Pf*PKA. Importantly, however, the compounds inhibited parasite cytokinesis and erythrocyte invasion at multiple steps indicating that the target, like *Pf*PKA, functions during invasion and is possibly another kinase. These compounds provide much needed new tools to provide insight into the mechanisms by which malaria parasites establish infection, particularly if their molecular targets can be identified. To pave the way for the optimisation of 4-cyano-3-methylisoquinolines as potential antimalarial agents they should be tested for toxicity against cultured human cells. This will be a particularly important consideration since compound **25** has shown some inhibition of four human kinases.

Experimental

Chemistry

All commercial materials were used as received without further purification. Glassware used in moisture sensitive reactions was oven dried and cooled under nitrogen prior to use and non-aqueous reactions performed under an atmosphere of nitrogen. All reactions were carried out at room temperature, unless otherwise noted. Purification of solvents and reagents, if required, was carried out by procedures described by Chai and Armarego.[50] Melting points were determined on a Reichert 'Thermopan' microscope hot stage apparatus as uncorrected values. All organic extracts were dried over magnesium sulfate unless otherwise stated. All reactions were monitored by Thin Layer Chromatography (TLC) unless otherwise noted. Analytical TLC was performed on Merck Kieselgel 60 F_{254} aluminium backed plates and visualised using a 254 nm UV lamp. Flash chromatography was performed on silica gel (Davisil® LC60 Å 40–63 micron) according to the method of Still *et al.*[51] Low-resolution electrospray ionisation (ESI) mass spectra were recorded on a Bruker Daltronics Esquire 6000 Ion Trap mass spectrometer in methanol or acetonitrile (0.1% formic acid) at 300 °C, a 40 eV cone voltage, with a scan rate of 5500 *m*/*z*/s. High-resolution ESI mass spectra were recorded on an Agilent 6224 TOF LC/MS coupled to an Agilent 1290 Infinity LC. All data was acquired and reference mass corrected *via* a dual-spray ESI source. Analytical Reverse-Phase High Performance Liquid Chromatography (RP-HPLC) was performed on a Shimadzu LC-20AB Prominence Liquid Chromatography system fitted with a Phenomenex® Jupiter C18 300 Å column (250 mm × 4.6 mm, 10 μm). Using either Method A: 10 → 90%

ACN in 0.1% TFA or Method B: 10 → 95% ACN in 10 mM NH_4HCO_3 over 25 minutes with a flow rate of 1 mL min^{-1}, monitored at 254 nm. Semi-preparative reverse phase HPLC was performed on a Beckman system (125 Solvent Module and 166 Detector) fitted with a Phenomenex® Jupiter C18 300 Å column (250 mm × 10.0 mm, 10 μm) at a flow rate of 5 mL min^{-1}, monitored at 254 nm. NMR spectra were recorded on either a Bruker AV-300, AV-400 and AV-500 spectrometer at 300.19, 400.13 and 500.02 MHz respectively, for ^{1}H nuclei and at 75.78, 100.62 and 125.74 MHz respectively, for ^{13}C nuclei at 300 K. For ^{1}H NMR the residual $CDCl_3$ peak (7.26 ppm) or DMSO-d_6 peak (2.50 ppm) were used as internal standards. Similarly, ^{13}C NMR spectra were referenced to the residual solvent; the central peak of the $CDCl_3$ 'triplet' (77.16 ppm) or DMSO-d_6 'heptet' (39.52 ppm). Chemical shifts were reported as δ values in parts per million (ppm). The following abbreviations have been used upon reporting spectral data: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; app, apparent; and br, broad. Coupling constants (J) are reported in Hz to the nearest 0.5 Hz.

General procedure A: preparation of substituted amides

***N*-(4-Cyano-3-methyl-1*H*-isochromen-1-ylidene)acetamide (6).** Prepared according to the original method of Gabriel and Neumann.[52] (Synthesis reported,[52] correct structure reported[29]). A mixture of 2-cyanophenylacetonitrile **5** (5.0 g, 35 mmol) and anhydrous sodium acetate (2.5 g) in distilled acetic anhydride (25 mL) was heated under reflux for 2 h. The resulting mixture was poured onto ice and stirred for 0.25 h. The solid that separated was filtered, washed with water and dried to give acetamide **6** (7.2 g, 91%) as a brown solid. The obtained material was sufficiently pure (>95% by ^{1}H-NMR) for further reaction. A portion was recrystallised from carbon tetrachloride to afford the amide as a light brown solid, mp 135–136 °C (lit.[52] 137–138 °C). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.13 (d, J = 8.0 Hz, 1H), 7.74–7.70 (m, 1H), 7.58 (d, J = 8.0 Hz, 1H), 7.53–7.49 (m, 1H), 2.50 (s, 3H), 2.31 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 182.7, 163.7, 145.9, 134.6, 130.0, 129.7, 128.2, 123.5, 119.8, 114.3, 92.7, 25.9, 19.5.

General procedure B1: preparation of 1-substituted 4-cyano-3-methylisoquinoline compounds *via* aminolysis

1-(Butylamino)-3-methylisoquinoline-4-carbonitrile (4). Prepared according to the method reported by Deady and Quazi.[29] A solution of acetamide **6** (0.50 g, 2.2 mmol) in acetone (7.4 mL) was added dropwise to a stirring solution of *n*-butylamine (7.4 mL, 75 mmol) in acetone (7.4 mL). After 0.5 h, ensuring the temperature remained <25 °C, the mixture was poured onto ice and the resulting solid that separated was filtered, washed with water, dried and recrystallised from carbon tetrachloride to afford the title compound **4** (0.41 g, 78%) as a pink solid, mp 135–137 °C (lit.[29] 136–138 °C). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.95 (dd, J = 9.0, 1.0 Hz, 1H), 7.72–7.68 (m, 2H), 7.50–7.46 (m, 1H), 5.65 (br s, 1H), 3.71–3.67 (m, 2H), 2.70 (s, 3H), 1.75–1.68 (m, 2H), 1.53–1.43 (m, 2H), 1.00 (t, J = 7.5 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 159.6, 156.1, 136.3, 131.5, 126.3, 124.7, 121.5, 118.7, 115.3, 91.9, 41.5, 31.7, 24.4, 20.4, 14.0. LRMS-ESI (m/z): 240.1 $(M + H)^+$. RP-HPLC: t_R 12.3 min (Method A).

1-(Butylamino)-3-propylisoquinoline-4-carbonitrile (9). 2-Cyanophenylacetonitrile **5** (0.50 g, 3.5 mmol) was treated according to General procedure A, except freshly distilled butanoic anhydride (3.2 mL) was used. The resulting butyramide intermediate **7** (0.93 g, 3.3 mmol) was coupled with *n*-butylamine (11 mL, 0.11 mol) according to General procedure B1, except the oil that separated was extracted with dichloromethane (×3). The combined organics were washed with brine (×2), dried, filtered and concentrated *in vacuo*. The crude solid was purified by flash chromatography (5% ethyl acetate in hexanes) and subsequent purification by semi-preparative reverse phase HPLC (10 → 90% acetonitrile in 0.1% trifluoroacetic acid over 120 min) provided compound **9** (10 mg, 1% over two steps) as a transparent solid, mp 117–119 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.97 (d, J = 8.0 Hz, 1H), 7.73–7.68 (m, 2H), 7.50–7.46 (m, 1H), 5.72 (br s, 1H), 3.72–3.67 (m, 2H), 2.97–2.94 (m, 2H), 1.91–1.82 (m, 2H), 1.75–1.67 (m, 2H), 1.52–1.42 (m, 2H), 1.04–0.97 (m, 6H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 163.1, 156.1, 136.2, 131.2, 126.1, 124.7, 121.5, 118.6, 115.3, 91.7, 41.3, 39.3, 31.5, 22.3, 20.2, 13.9, 13.8. LRMS-ESI (m/z): 268.2 $(M + H)^+$. HRMS-ESI (m/z): $(M + H)^+$ calcd for $C_{17}H_{22}N_3^+$, 268.1808; found, 268.1808. RP-HPLC: t_R 15.5 min (Method A).

1-(Butylamino)-3-pentylisoquinoline-4-carbonitrile (10). 2-Cyanophenylacetonitrile **5** (0.50 g, 3.5 mmol) was treated according to General procedure A, except freshly distilled hexanoic anhydride (4.4 mL) was used. The resulting hexanamide intermediate **8** (0.96 g, 2.8 mmol) was coupled with *n*-butylamine (9.5 mL, 97 mmol) according to General procedure B1, except the oil that separated was extracted with ethyl acetate (×3). The combined organics were washed with brine (×2), dried, filtered and concentrated *in vacuo*. The crude solid was purified by flash chromatography (10% ethyl acetate in hexanes) to provide compound **10** (60 mg, 7% over two steps) as a white solid, mp 114–115 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.96 (d, J = 8.0 Hz, 1H), 7.73–7.66 (m, 2H), 7.49–7.45 (m, 1H), 5.74 (br s, 1H), 3.72–3.67 (m, 2H), 2.97 (t, J = 7.5 Hz, 2H), 1.87–1.79 (m, 2H), 1.75–1.67 (m, 2H), 1.51–1.43 (m, 2H), 1.41–1.37 (m, 4H), 0.99 (t, J = 7.5 Hz, 3H), 0.90 (t, J = 7.5 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 163.4, 156.1, 136.3, 131.3, 126.1, 124.7, 121.4, 118.6, 115.3, 91.5, 41.3, 37.3, 31.6, 31.4, 28.7, 22.6, 20.3, 14.0, 13.9. LRMS-ESI (m/z): 296.2 $(M + H)^+$. HRMS-ESI (m/z): $(M + H)^+$ calcd for $C_{19}H_{26}N_3^+$, 296.2121; found, 296.2123. RP-HPLC: t_R 23.6 min (Method B).

1-Amino-3-methylisoquinoline-4-carbonitrile (11). Prepared according to the method of Deady and Quazi.[29] A solution of acetamide **6** (0.20 g, 0.88 mmol) and ammonia (10 mL, 30% aqueous, d 0.89) in dioxane (10 mL) was heated under reflux for 1 h. Water was added and the solid that separated was filtered, washed with water and recrystallised from ethanol to afford amine **11** (0.15 g, 93%) as a light pink solid, mp 273–274 °C (lit.[29] 273–274 °C). ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.29 (d, J = 8.5 Hz, 1H), 7.88 (br s, 2H), 7.81–7.77 (m, 1H), 7.73 (dd, J = 8.5, 1.0 Hz, 1H), 7.56–7.52 (m, 1H), 2.54 (s, 3H).

^{13}C NMR (100 MHz, DMSO-d_6): δ 159.3, 158.9, 135.8, 132.3, 126.0, 124.7, 122.8, 118.2, 114.5, 89.5, 23.4. LRMS-ESI (m/z): 184.0 $(M + H)^+$. RP-HPLC: t_R 8.5 min (Method A).

General procedure B2: preparation of 1-substituted 4-cyano-3-methylisoquinoline compounds *via* aminolysis using an acidic workup

1-(Hexylamino)-3-methylisoquinoline-4-carbonitrile (12). To a stirring solution of *n*-hexylamine (4.0 mL, 30 mmol) in acetone (4.0 mL), a solution of acetamide **6** (0.20 g, 0.88 mmol) in acetone (4.0 mL) was added dropwise ensuring the reaction temperature was maintained <25 °C. After stirring at room temperature for 0.5 h, the mixture was poured onto ice and the oil separated was extracted with dichloromethane (×3). The combined organics were washed with brine (×2), dried, filtered and concentrated *in vacuo* to give a brown oil residue. Water was added and the mixture neutralised with 3 M hydrochloric acid or until a solid precipitated and the mixture was subsequently kept overnight at 4 °C. The crude product was purified by flash chromatography (10% ethyl acetate in hexanes) and subsequently recrystallised from toluene/petroleum ether (bp 80–110 °C) to give the title compound **12** (86 mg, 36%) as a white solid, mp 113–115 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.94 (d, J = 8.5 Hz, 1H), 7.74–7.66 (m, 2H), 7.50–7.45 (m, 1H), 5.72 (br s, 1H), 3.71–3.64 (m, 2H), 2.70 (s, 3H), 1.77–1.67 (m, 2H), 1.47–1.33 (m, 6H), 0.90 (t, J = 7.0 Hz, 3H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 159.6, 156.0, 136.3, 131.6, 126.3, 124.7, 121.6, 118.7, 115.3, 91.9, 41.8, 31.7, 29.5, 26.9, 24.3, 22.7, 14.2. LRMS-ESI (m/z): 268.1 $(M + H)^+$. HRMS-ESI (m/z): $(M + H)^+$ calcd for $C_{17}H_{22}N_3^+$, 268.1808; found, 268.1809. RP-HPLC: t_R 14.8 min (Method A).

3-Methyl-1-(octylamino)isoquinoline-4-carbonitrile (13). Acetamide **6** (0.50 g, 2.2 mmol) was coupled with *n*-octylamine (12 mL, 75 mmol) according to General procedure B2. The crude product was recrystallised from ethanol to afford the title compound **13** (99 mg, 15%) as a light pink solid, mp 106–109 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.32 (d, J = 8.5 Hz, 1H), 8.29 (br t, J = 5.5 Hz, 1H), 7.80–7.72 (m, 2H), 7.57–7.53 (m, 1H), 3.58–3.53 (m, 2H), 2.56 (s, 3H), 1.67–1.61 (m, 2H), 1.35–1.24 (m, 10H), 0.84 (t, J = 7.0 Hz, 3H). ^{13}C NMR (100 MHz, DMSO-d_6): δ 158.9, 156.3, 135.3, 131.9, 126.1, 123.7, 122.9, 118.4, 115.1, 89.1, 40.8, 31.2, 28.7, 28.6, 28.3, 26.5, 23.9, 22.1, 13.9. LRMS-ESI (m/z): 296.2 $(M + H)^+$. HRMS-ESI (m/z): $(M + H)^+$ calcd for $C_{19}H_{26}N_3^+$, 296.2121; found, 296.2122. RP-HPLC: t_R 17.3 min (Method A).

1-(2-Propylamino)-3-methylisoquinoline-4-carbonitrile (14). Acetamide **6** (2.0 g, 8.9 mmol) was coupled with 2-propylamine (26 mL, 0.30 mol) according to General procedure B1. The crude product was recrystallised from carbon tetrachloride to afford the title compound **14** (1.4 g, 70%) as a pale brown solid, mp 203–206 °C. ^{1}H NMR (300 MHz, DMSO-d_6): δ 8.40 (d, J = 8.0 Hz, 1H), 7.96 (d, J = 7.5 Hz, 1H), 7.83–7.71 (m, 2H), 7.58–7.52 (m, 1H), 4.63–4.52 (m, 1H), 2.56 (s, 3H), 1.27 (d, J = 6.5 Hz, 6H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 159.0, 155.6, 135.4, 131.9, 126.0, 123.9, 122.9, 118.4, 115.1, 89.0, 42.3, 24.0, 22.0. LRMS-ESI (m/z): 226.1 $(M + H)^+$. HRMS-ESI (m/z): $(M + H)^+$ calcd for $C_{14}H_{16}N_3^+$, 226.1339; found, 226.1339. RP-HPLC: t_R 10.2 min (Method A).

1-(2-Methylpropylamino)-3-methylisoquinoline-4-carbonitrile (15). Acetamide **6** (0.50 g, 2.2 mmol) was coupled with 2-methylpropylamine (7.5 mL, 75 mmol) according to General procedure B1. The crude product was recrystallised from toluene to afford the title compound **15** (0.30 g, 57%) as a light pink solid, mp 147–149 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.95 (d, J = 8.0 Hz, 1H), 7.74–7.67 (m, 2H), 7.51–7.45 (m, 1H), 5.78 (br s, 1H), 3.53 (dd, J = 7.0, 5.5 Hz, 2H), 2.69 (s, 3H), 2.11–1.97 (m, 1H), 1.04 (d, J = 6.5 Hz, 6H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 159.6, 156.2, 136.4, 131.6, 126.3, 124.8, 121.5, 118.7, 115.3, 92.0, 49.1, 28.5, 24.4, 20.5. LRMS-ESI (m/z): 240.0 $(M + H)^+$. HRMS-ESI (m/z): $(M + H)^+$ calcd for $C_{15}H_{18}N_3^+$, 240.1495; found, 240.1496. RP-HPLC: t_R 11.8 min (Method A).

3-Methyl-1-(2,2-dimethylpropanamino)isoquinoline-4-carbonitrile (16). Acetamide **6** (0.30 g, 1.3 mmol) was coupled with 2,2-dimethyl-1-propanamine (5.3 mL, 45 mmol) according to General procedure B1, except the oil that separated was extracted with dichloromethane (×3). The combined organics were washed with brine (×2), dried, filtered and concentrated *in vacuo*. The crude solid was recrystallised from toluene to give compound **16** (0.18 g, 54%) as a white solid, mp 176–178 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.94 (d, J = 8.0 Hz, 1H), 7.75–7.67 (m, 2H), 7.52–7.46 (m, 1H), 5.79 (br s, 1H), 3.57 (d, J = 6.0 Hz, 2H), 2.69 (s, 3H), 1.04 (s, 9H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 159.5, 156.4, 136.4, 131.6, 126.4, 124.8, 121.3, 118.7, 115.3, 91.9, 52.4, 32.4, 27.7, 24.3. LRMS-ESI (m/z): 254.1 $(M + H)^+$. HRMS-ESI (m/z): $(M + H)^+$ calcd for $C_{16}H_{20}N_3^+$, 254.1652; found, 254.1652. RP-HPLC: t_R 13.8 min (Method A).

(±)-3-Methyl-1-((2-methylbutyl)amino)isoquinoline-4-carbonitrile (17). Acetamide **6** (0.30 g, 1.3 mmol) was coupled with (±)-2-methylbutylamine (5.3 mL, 45 mmol) according to General procedure B2. The crude product was recrystallised from toluene to afford the title compound **17** (0.13 g, 39%) as an off-white solid, mp 130–132 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.96 (dd, J = 8.0, 1.5 Hz, 1H), 7.72–7.68 (m, 2H), 7.51–7.46 (m, 1H), 5.72 (br s, 1H), 3.69–3.47 (m, 2H), 2.70 (s, 3H), 1.85–1.75 (m, 1H), 1.56–1.47 (m, 1H), 1.34–1.23 (m, 1H), 1.02 (d, J = 7.0 Hz, 3H), 0.97 (t, J = 7.0 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 159.6, 156.2, 136.4, 131.6, 126.3, 124.8, 121.4, 118.7, 115.3, 91.9, 47.3, 35.0, 27.4, 24.4, 17.7, 11.5. LRMS-ESI (m/z): 254.0 $(M + H)^+$. HRMS-ESI (m/z): $(M + H)^+$ calcd for $C_{16}H_{20}N_3^+$, 254.1652; found, 254.1652. RP-HPLC: t_R 13.4 min (Method A).

1-(Isopentylamino)-3-methylisoquinoline-4-carbonitrile (18). Acetamide **6** (0.20 g, 0.88 mmol) was coupled with isopentylamine (3.5 mL, 30 mmol) according to General procedure B2. Purification by flash chromatography (10% ethyl acetate in hexanes) and subsequent recrystallisation from toluene/petroleum ether (bp 80–110 °C) gave the title compound **18** (32 mg, 14%) as a light pink solid, mp 149–151 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.32 (d, J = 8.5 Hz, 1H), 8.27 (br t, J = 5.5 Hz, 1H), 7.81–7.72 (m, 2H), 7.57–7.53 (m, 1H), 3.62–3.57 (m, 2H), 2.56 (s, 3H), 1.71–1.61 (m, 1H), 1.58–1.52 (m, 2H), 0.94 (d, J = 6.5 Hz, 6H). ^{13}C NMR (100 MHz, DMSO-d_6): δ 159.0, 156.3, 135.3, 131.9, 126.1, 123.7, 123.0, 118.4, 115.2,

89.0, 39.1 (obscured by DMSO peak), 37.5, 25.4, 24.0, 22.5. LRMS-ESI (*m*/*z*): 254.1 $(M + H)^+$. HRMS-ESI (*m*/*z*): $(M + H)^+$ calcd for $C_{16}H_{20}N_3^+$, 254.1652; found, 254.1653. RP-HPLC: t_R 13.5 min (Method A).

(±)-3-Methyl-1-((4-methyl-2-pentyl)amino)isoquinoline-4-carbonitrile (19). Acetamide **6** (85 mg, 0.38 mmol) was coupled with (±)-4-methyl-2-pentylamine (1.8 mL, 13 mmol) according to General procedure B2. Purification by flash chromatography (10% ethyl acetate in hexanes) provided the title compound **19** (22 mg, 22%) as a pale yellow semi-solid, mp 78–83 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.94 (dd, J = 8.5, 1.0 Hz, 1H), 7.71–7.67 (m, 2H), 7.49–7.45 (m, 1H), 5.40 (d, J = 7.5 Hz, 1H), 4.69–4.62 (m, 1H), 2.69 (s, 3H), 1.76–1.65 (m, 1H), 1.61–1.37 (m, 2H), 1.29 (d, J = 6.5 Hz, 3H), 0.97 (d, J = 5.5 Hz, 3H), 0.95 (d, J = 5.5 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 159.7, 155.5, 136.5, 131.5, 126.2, 124.7, 121.5, 118.8, 115.2, 91.6, 46.9, 45.2, 25.4, 24.4, 23.0, 22.9, 21.5. LRMS-ESI (*m*/*z*): 268.1 $(M + H)^+$. HRMS-ESI (*m*/*z*): $(M + H)^+$ calcd for $C_{17}H_{22}N_3^+$, 268.1808; found, 268.1808. RP-HPLC: t_R 14.9 min (Method A).

(±)-1-((5-(Diethylamino)2-pentyl)amino)-3-methylisoquinoline-4-carbonitrile (20). Acetamide **6** (0.20 g, 0.88 mmol) was coupled with (±)-5-diethylamino-2-pentylamine (5.8 mL, 30 mmol) according to General procedure B1, except the reaction mixture was not poured onto ice. The reaction mixture was concentrated *in vacuo* and the excess amine was distilled off to give a brown residue. The crude residue was dissolved in ethanol, to which a drop of phosphoric acid was added, followed by diethyl ether (2 mL) and the solvent subsequently decanted. Following the addition of water (5 mL) the suspension was basified with 25% sodium hydroxide and the whole extracted with dichloromethane (×3). The combined organics were washed with brine (×2), dried, filtered and concentrated *in vacuo* to afford compound **20** (54 mg, 19%) as a green oil, mp 31–35 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.89 (d, J = 8.5 Hz, 1H), 7.84 (d, J = 8.5 Hz, 1H), 7.68–7.64 (m, 1H), 7.47–7.42 (m, 1H), 6.21 (d, J = 8.0 Hz, 1H), 4.57–4.50 (m, 1H), 2.66 (s, 3H), 2.58 (q, J = 7.0 Hz, 4H), 2.51 (td, J = 7.0, 2.5 Hz, 2H), 1.80–1.58 (m, 4H), 1.30 (d, J = 6.5 Hz, 3H), 1.03 (t, J = 7.0 Hz, 6H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 159.6, 155.8, 136.4, 131.4, 126.1, 124.5, 122.1, 118.9, 115.4, 91.4, 52.8, 47.0, 46.8, 34.7, 24.4, 23.5, 20.9, 11.1. LRMS-ESI (*m*/*z*): 325.2 $(M + H)^+$. HRMS-ESI (*m*/*z*): $(M + 2H)^{2+}$ calcd for $C_{20}H_{29}N_4^+$, 163.1230; found, 163.1227. RP-HPLC: t_R 9.45 min (Method A).

3-Methyl-1-morpholinoisoquinoline-4-carbonitrile (21). Acetamide **6** (0.30 g, 1.3 mmol) was coupled with morpholine (3.9 mL, 45 mmol) according to General procedure B2. The crude product was recrystallised from toluene/petroleum ether (bp 80–110 °C) to afford the title compound **21** (95 mg, 28%) as a deep orange solid, mp 158–161 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.03 (d, J = 8.5 Hz, 1H), 7.98 (d, J = 8.5 Hz, 1H), 7.76–7.70 (m, 1H), 7.54–7.48 (m, 1H), 3.92 (t, J = 5.0 Hz, 4H), 3.64 (t, J = 5.0 Hz, 4H), 2.75 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 161.8, 157.5, 138.0, 131.7, 126.3, 126.0, 124.7, 117.8, 117.7, 96.8, 67.0, 51.5, 24.1. LRMS-ESI (*m*/*z*): 254.0 $(M + H)^+$. HRMS-ESI (*m*/*z*): $(M + H)^+$ calcd for $C_{15}H_{16}N_3O^+$, 254.1288; found, 254.1292. RP-HPLC: t_R 15.8 min (Method A).

(*R*)-3-Methyl-1-((1-phenylethyl)amino)isoquinoline-4-carbonitrile (22). Acetamide **6** (0.26 g, 1.2 mmol) was coupled with (*R*)-(+)-1-phenylethylamine (5.0 mL, 39 mmol) according to General procedure B2. The crude product was recrystallised from ethanol to afford the title compound **22** (0.14 g, 42%) as a lime green solid, mp 152–154 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.55 (d, J = 8.5 Hz, 1H), 8.52 (d, J = 7.5 Hz, 1H), 7.83–7.79 (m, 1H), 7.74 (dd, J = 8.0, 1.0 Hz, 1H), 7.61–7.57 (m, 1H), 7.47–7.44 (m, 2H), 7.32–7.28 (m, 2H), 7.22–7.18 (m, 1H), 5.71–5.63 (m, 1H), 2.51 (s, 3H), 1.60 (d, J = 7.0 Hz, 3H). ^{13}C NMR (100 MHz, DMSO-d_6): δ 158.7, 155.4, 144.7, 135.4, 132.1, 128.2, 126.6, 126.3, 126.2, 124.0, 122.9, 118.2, 115.0, 89.7, 49.5, 23.9, 21.9. LRMS-ESI (*m*/*z*): 288.1 $(M + H)^+$. HRMS-ESI (*m*/*z*): $(M + H)^+$ calcd for $C_{19}H_{18}N_3^+$, 288.1495; found, 288.1495. RP-HPLC: t_R 16.8 min (Method A).

(*S*)-3-Methyl-1-((1-phenylethyl)amino)isoquinoline-4-carbonitrile (23). Acetamide **6** (0.50 g, 2.2 mmol) was coupled with (*S*)-(−)-1-phenylethylamine (9.7 mL, 75 mmol) according to General procedure B2, except after pH adjustment, the supernatant was decanted off and the residual solid was suspended in water. The solid was extracted with dichloromethane (×3), washed with brine (×2), dried, filtered and concentrated *in vacuo* to give a brown oil residue. The residue was recrystallised from ethanol to give the title compound **23** (0.34 g, 54%) as a pink solid, mp 151–154 °C. ^{1}H NMR (300 MHz, DMSO-d_6): δ 8.54 (d, J = 8.0 Hz, 1H), 8.51 (d, J = 8.0 Hz, 1H), 7.82–7.71 (m, 2H), 7.61–7.55 (m, 1H), 7.47–7.44 (m, 2H), 7.33–7.28 (m, 2H), 7.22–7.17 (m, 1H), 5.71–5.62 (m, 1H), 2.51 (s, 3H), 1.60 (d, J = 7.0 Hz, 3H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 158.6, 155.4, 144.7, 135.4, 132.0, 128.1, 126.5, 126.3, 126.1, 124.0, 122.9, 118.1, 115.0, 89.7, 49.5, 23.8, 21.9. LRMS-ESI (*m*/*z*): 288.1 $(M + H)^+$. HRMS-ESI (*m*/*z*): $(M + H)^+$ calcd for $C_{19}H_{18}N_3^+$, 288.1495; found, 288.1495. RP-HPLC: t_R 16.8 min (Method A).

1-(Benzhydrylamino)-3-methylisoquinoline-4-carbonitrile (24). Acetamide **6** (0.18 g, 0.80 mmol) was coupled with benzhydrylamine (4.7 mL, 27 mmol) according to General procedure B1. Upon pouring the reaction mixture onto ice the supernatant was decanted off. The crude mixture was boiled in ethanol and the off-white solid that separated upon cooling was filtered. The crude product was purified by flash chromatography (2.5–5% ethyl acetate in hexanes) and subsequent recrystallisation from ethanol provided compound **24** (35 mg, 13%) as a white solid, mp 194–196 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.91 (d, J = 8.5 Hz, 1H), 8.67 (d, J = 8.5 Hz, 1H), 7.85–7.76 (m, 2H), 7.62–7.57 (m, 1H), 7.42–7.33 (m, 8H), 7.29–7.25 (m, 2H), 6.98 (d, J = 8.5 Hz, 1H), 2.55 (s, 3H). ^{13}C NMR (100 MHz, DMSO-d_6): δ 158.5, 155.5, 142.0, 135.5, 132.2, 128.3, 127.9, 127.0, 126.3, 124.3, 123.0, 118.0, 115.1, 90.5, 57.3, 23.9. LRMS-ESI (*m*/*z*): 350.1 $(M + H)^+$. HRMS-ESI (*m*/*z*): $(M + H)^+$ calcd for $C_{24}H_{20}N_3^+$, 350.1652; found, 350.1655. RP-HPLC: t_R 21.2 min (Method A).

3-Methyl-1-(1-ethylpropylamino)isoquinoline-4-carbonitrile (25). Acetamide **6** (1.0 g, 4.4 mmol) was coupled with 1-ethylpropylamine (18 mL, 0.15 mol) according to General procedure B2. Purification by flash chromatography (10% ethyl acetate in hexanes) and subsequent recrystallisation from toluene/

petroleum ether (bp 80–110 °C) afforded the title compound **25** (0.89 g, 79%) as a light brown solid, mp 102–104 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.95 (d, J = 8.0 Hz, 1H), 7.73–7.67 (m, 2H), 7.49–7.45 (m, 1H), 5.42 (br d, J = 8.0 Hz, 1H), 4.46–4.38 (m, 1H), 2.68 (s, 3H), 1.80–1.54 (m, 4H), 0.96 (t, J = 7.5 Hz, 6H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 159.7, 156.1, 136.5, 131.5, 126.2, 124.8, 121.4, 118.8, 115.2, 91.6, 53.4, 27.2, 24.4, 10.4. LRMS-ESI (m/z): 254.1 $(M + H)^+$. HRMS-ESI (m/z): $(M + H)^+$ calcd for $C_{16}H_{20}N_3^+$, 254.1652; found, 254.1651. RP-HPLC: t_R 12.9 min (Method A).

1-(2-Methylpropylamino)-3-methylisoquinoline-4-carboxamide (26). A solution of **15** (0.10 g, 0.42 mmol) in 1 : 1 concentrated sulfuric acid/water (4 mL) was heated under reflux for 65 h. The solution was then cooled, poured onto ice and adjusted to pH 9 with 25% sodium hydroxide. The whole was extracted with dichloromethane (×3), washed with brine (×2), dried, filtered and concentrated *in vacuo*. Purification by flash chromatography (15 → 80% ethyl acetate in hexanes) afforded amide **26** (27 mg, 25%) as a light brown solid, mp 157–159 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.24 (d, J = 8.5 Hz, 1H), 7.72 (br s, 1H), 7.65–7.57 (m, 2H), 7.47 (br s, 1H), 7.44–7.39 (m, 2H), 3.32 (obscured by H_2O peak), 2.36 (s, 3H), 2.12–2.02 (m, 1H), 0.93 (d, J = 6.5 Hz, 6H). ^{13}C NMR (100 MHz, DMSO-d_6): δ 170.7, 154.5, 145.4, 134.4, 129.7, 124.4, 123.9, 122.8, 116.8, 115.4, 48.4, 27.2, 22.8, 20.5. LRMS-ESI (m/z): 258.0 $(M + H)^+$. HRMS-ESI (m/z): $(M + H)^+$ calcd for $C_{15}H_{20}N_3O^+$, 258.1601; found, 258.1600. RP-HPLC: t_R 7.53 min (Method A).

1-Ethoxy-3-methylisoquinoline-4-carbonitrile (27). Acetamide **6** (0.20 g, 0.88 mmol) and potassium ethoxide (0.19 g, 2.2 mmol) was dissolved in anhydrous ethanol (25 mL) and the reaction was heated under reflux for 1 h. The mixture was then concentrated *in vacuo*, water added (25 mL), neutralised with 3 M hydrochloric acid and the whole extracted with chloroform (×3). The combined organic extracts were washed with brine (×2), dried, filtered and concentrated *in vacuo.* The crude solid was recrystallised from ethanol to give ethoxide **27** (13 mg, 7%) as an orange solid, mp 113–115 °C (lit.[29] 114–116 °C). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.27 (ddd, J = 8.0, 1.5, 0.5 Hz, 1H), 7.99 (ddd app dt, J = 8.0, 1.0 Hz, 1H), 7.81–7.76 (m, 1H), 7.59–7.57 (m, 1H), 4.63 (q, J = 7.0 Hz, 2H), 2.77 (s, 3H), 1.51 (t, J = 7.0 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 162.1, 158.0, 137.2, 132.5, 127.2, 124.9, 123.7, 117.4, 117.4, 97.2, 63.2, 24.0, 14.6. LRMS-ESI (m/z): 213.0 $(M + H)^+$. RP-HPLC: t_R 20.9 min (Method A).

1-Butoxy-3-methylisoquinoline-4-carbonitrile (28). Sodium in anhydrous *n*-butanol (25 mL), under nitrogen, was heated to complete dissolution and then allowed to cool to room temperature. To this acetamide **6** (0.20 g, 0.88 mmol) was added and the reaction heated under reflux for 1 h. The mixture was then concentrated *in vacuo*, water added (25 mL), neutralised with 3 M hydrochloric acid and the whole extracted with dichloromethane (×3). The combined organic extracts were washed with brine (×2), dried, filtered and concentrated *in vacuo*. The crude solid obtained was recrystallised from *n*-butanol to give the butoxide **28** (55 mg, 26%) as an off-white solid, mp 80–83 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.26 (d, J = 8.5 Hz, 1H), 7.99 (d, J = 8.5 Hz, 1H), 7.81–7.76 (m, 1H), 7.59–7.55 (m, 1H), 4.57 (t, J = 6.5 Hz, 2H), 2.77 (s, 3H), 1.91–1.84 (m, 2H), 1.59–1.53 (m, 2H), 1.02 (t, J = 7.5 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 162.2, 158.1, 137.2, 132.5, 127.3, 124.9, 123.7, 117.5, 117.4, 97.2, 67.2, 31.1, 24.0, 19.5, 14.0. LRMS-ESI (m/z): 241.1 $(M + H)^+$. HRMS-ESI (m/z): $(M + H)^+$ calcd for $C_{15}H_{17}N_2O^+$, 241.1335; found, 241.1335. RP-HPLC: t_R 23.6 min (Method A).

General procedure C: preparation of 7-bromo-4-cyano-3-methylisoquinoline compounds *via* bromination using *N*-bromosuccinimide

1-Amino-7-bromo-3-methylisoquinoline-4-carbonitrile (29). Isoquinoline **11**[29] (0.10 g, 0.55 mmol) was added portion wise to ice cold concentrated sulfuric acid (2 mL). Once the solid was dissolved, *N*-bromosuccinimide (0.12 g, 0.66 mmol) was added in portions and the reaction mixture stirred at 0 °C for 0.5 h, then allowed to come to room temperature and stirred for a further 2 h. The reaction mixture was poured onto ice, adjusted to pH 5 with 25% sodium hydroxide and the solid that separated was filtered and washed with water. The crude product was purified by flash chromatography (70% ethyl acetate in hexanes) to afford compound **29** (82 mg, 57%) as a light brown solid, mp 255–260 °C (dec). ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.65 (d, J = 2.0 Hz, 1H), 8.21 (br s, 2H), 7.98 (dd, J = 9.0, 2.0 Hz, 1H), 7.71 (d, J = 9.0 Hz, 1H), 2.54 (s, 3H). ^{13}C NMR (100 MHz, DMSO-d_6): δ 159.7, 156.0, 137.3, 133.9, 127.9, 125.7, 120.3, 116.8, 116.1, 91.5, 21.0. LRMS-ESI (m/z): 262.0 $(M[^{79}Br] + H)^+$, 264.0 $(M[^{81}Br] + H)^+$. HRMS-ESI (m/z): $(M[^{79}Br] + H)^+$ calcd for $C_{11}H_9BrN_3^+$, 261.9974; found, 261.9977. RP–HPLC: t_R 10.2 min (Method A).

7-Bromo-1-(2-propylamino)-3-methylisoquinoline-4-carbonitrile (30). Isoquinoline **14** (0.30 g, 1.3 mmol) was brominated according to General procedure C, except was further kept overnight at 4 °C prior to filtering. The crude product recrystallised from methanol to afford compound **30** (0.14 g, 35%) as a brown solid, mp 177–179 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.72 (d, J = 2.0 Hz, 1H), 8.09 (br d, J = 7.0 Hz, 1H), 7.92 (dd, J = 9.0, 2.0 Hz, 1H), 7.66 (d, J = 9.0 Hz, 1H), 4.59–4.51 (m, 1H), 2.55 (s, 3H), 1.26 (d, J = 6.5 Hz, 6H). ^{13}C NMR (100 MHz, DMSO-d_6): δ 159.5, 154.6, 134.8, 134.3, 126.3, 125.2, 118.8, 118.0, 116.4, 88.8, 42.5, 24.0, 21.9. LRMS-ESI (m/z): 303.9 $(M[^{79}Br] + H)^+$, 305.9 $(M[^{81}Br] + H)^+$. HRMS-ESI (m/z): $(M[^{79}Br] + H)^+$ calcd for $C_{14}H_{15}BrN_3^+$, 304.0444; found, 304.0444. RP-HPLC: t_R 16.0 min (Method A).

7-Bromo-1-(butylamino)-3-methylisoquinoline-4-carbonitrile (31). Isoquinoline **4** (2.0 g, 8.4 mmol) was brominated according to General procedure C. The crude product was recrystallised from methanol to afford compound **31** (1.9 g, 70%) as a beige solid, mp 176–178 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.62 (d, J = 2.0 Hz, 1H), 8.34 (br t, J = 5.0 Hz, 1H), 7.89 (dd, J = 9.0, 2.0 Hz, 1H), 7.63 (d, J = 9.0 Hz, 1H), 3.55–3.51 (m, 2H), 2.53 (s, 3H), 1.66–1.59 (m, 2H), 1.41–1.32 (m, 2H), 0.92 (t, J = 7.0 Hz, 3H). ^{13}C NMR (100 MHz, DMSO-d_6): δ 159.5, 155.4, 134.7, 134.2, 126.2, 125.2, 118.8, 117.9, 116.4, 88.9, 40.7, 30.4, 24.0, 19.7, 13.7. LRMS-ESI (m/z): 318.0 $(M[^{79}Br] + H)^+$, 320.0 (M

[^{81}Br] + H)$^+$. HRMS-ESI (*m*/*z*): (M[^{79}Br] + H)$^+$ calcd for $C_{15}H_{17}BrN_3^+$, 318.0600; found, 318.0600. RP-HPLC: t_R 17.4 min (Method A).

General procedure D: preparation of 7-substituted 4-cyano-3-methylisoquinoline *via* boronic acid coupling

1-(Butylamino)-3-methyl-7-phenylisoquinoline-4-carbonitrile (32). To a solution of **31** (0.20 g, 0.63 mmol) in 4 : 1 acetonitrile : water (50 mL), phenylboronic acid (0.12 g, 0.95 mmol) and caesium fluoride (0.29 g, 1.9 mmol) were added and the reaction mixture degassed under nitrogen. To this 1,1′ bis(diphenylphosphino)ferrocene]dichloropalladium (II) (23 mg, 5 mol%) was added and the reaction mixture was heated to 90 °C for 24 h. The mixture was then cooled, brine was added and the whole extracted with ethyl acetate (×2). The organic extracts were dried, filtered and concentrated *in vacuo* to give a brown solid. The crude solid was purified by flash chromatography (10% ethyl acetate in hexanes) to provide compound **32** (81 mg, 41%) as a white solid, mp 193–196 °C. ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.99 (dd, J = 8.5, 0.5 Hz, 1H), 7.91 (dd, J = 8.5, 2.0 Hz, 1H), 7.85 (d, J = 2.0 Hz, 1H), 7.66–7.63 (m, 2H), 7.51–7.47 (m, 2H), 7.42–7.39 (m, 1H), 5.84 (br s, 1H), 3.73–3.69 (m, 2H), 2.71 (s, 3H), 1.76–1.70 (m, 2H), 1.52–1.45 (m, 2H), 1.00 (t, J = 7.0 Hz, 3H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 159.4, 156.1, 140.1, 139.4, 135.3, 130.9, 129.1, 127.9, 127.3, 125.1, 119.6, 118.6, 115.5, 91.6, 41.5, 31.6, 24.2, 20.3, 13.9. LRMS-ESI (*m*/*z*): 316.2 (M + H)$^+$. HRMS-ESI (*m*/*z*): (M + H)$^+$ calcd for $C_{21}H_{22}N_3^+$, 316.1808; found, 316.1809. RP-HPLC: t_R 22.7 min (Method B).

1-(Butylamino)-7-(3-methoxyphenyl)-3-methylisoquinoline-4-carbonitrile (33). Compound **31** (0.10 g, 0.32 mmol) was coupled to 3-methoxyphenylboronic acid (72 mg, 0.47 mmol) according to General procedure D. The crude solid was purified by flash chromatography (10% ethyl acetate in hexanes) and subsequently recrystallised from petroleum ether (bp 80–110 °C) to provide compound **33** (39 mg, 36%) as a white solid, mp 161–166 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.98 (d, J = 9.0 Hz, 1H), 7.90 (dd, J = 9.0, 1.5 Hz, 1H), 7.83 (d, J = 1.5 Hz, 1H), 7.41 (dd app t, J = 8.0 Hz, 1H), 7.23 (ddd, J = 8.0, 2.0, 1.0 Hz, 1H), 7.17 (dd app t, J = 2.0 Hz, 1H), 6.95 (ddd, J = 8.0, 2.0, 1.0 Hz, 1H), 5.80 (br s, 1H), 3.89 (s, 3H), 3.75–3.68 (m, 2H), 2.71 (s, 3H), 1.77–1.69 (m, 2H), 1.53–1.43 (m, 2H), 1.00 (t, J = 7.5 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 160.2, 159.4, 156.0, 141.6, 139.2, 135.4, 130.9, 130.1, 125.1, 119.8, 119.6, 118.6, 115.4, 113.5, 112.9, 91.7, 55.5, 41.5, 31.6, 24.2, 20.3, 13.9. LRMS-ESI (*m*/*z*): 346.2 (M + H)$^+$. HRMS-ESI (*m*/*z*): (M + H)$^+$ calcd for $C_{22}H_{24}N_3O^+$, 346.1914; found, 346.1916. RP-HPLC: t_R 16.9 min (Method A).

1-(Butylamino)-3-methyl-7-(phenylamino)isoquinoline-4-carbonitrile (34). A mixture of **31** (0.10 g, 0.32 mmol), aniline (35 μL, 0.38 mmol) and potassium carbonate (96 mg, 0.69 mmol) in anhydrous *tert*-butanol (10 mL) was degassed under nitrogen. To this mixture 2-dicyclohexylphosphino-2′,4′,6′-triisopropylbiphenyl (15 mg, 10 mol%) and tris(dibenzylideneacetone)dipalladium(0) (14 mg, 5 mol%) were added and the reaction heated under reflux for 17 h. The mixture was then allowed to cool, water was added and the whole extracted with dichloromethane (×2). The combined organic extracts were washed with brine (×2), dried, filtered and concentrated *in vacuo* to give a brown oil. The crude oil was purified by flash chromatography (10% ethyl acetate in hexanes) to provide compound **34** (38 mg, 36%) as a yellow powder, mp 240–245 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.84 (d, J = 9.0 Hz, 1H), 7.41 (dd, J = 9.0, 2.0 Hz, 1H), 7.37–7.32 (m, 2H), 7.28 (d, J = 2.0 Hz, 1H), 7.16–7.13 (m, 2H), 7.06–7.02 (m, 1H), 5.93 (s, 1H), 5.38 (s, 1H), 3.67–3.62 (m, 2H), 2.67 (s, 3H), 1.71–1.63 (m, 2H), 1.49–1.40 (m, 2H), 0.98 (t, J = 7.5 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 156.8, 154.9, 142.2, 142.0, 130.7, 129.7, 126.0 124.1, 122.3, 118.8, 118.7, 116.3, 106.0, 91.7, 41.4, 31.5, 23.9, 20.3, 13.9. LRMS-ESI (*m*/*z*): 331.2 (M + H)$^+$. HRMS-ESI (*m*/*z*): (M + H)$^+$ calcd for $C_{21}H_{23}N_4^+$, 331.1917; found, 331.1921. RP-HPLC: t_R 21.1 min (Method B).

1,7-Bis(butylamino)-3-methylisoquinoline-4-carbonitrile (35). A mixture of **31** (0.20 g, 0.63 mmol), *n*-butylamine (75 μL, 0.76 mmol) and potassium carbonate (0.19 g, 1.4 mmol) in anhydrous *tert*-butanol (20 mL) was degassed under nitrogen. To this mixture 2-dicyclohexylphosphino-2′,4′,6′-triisopropylbiphenyl (60 mg, 20 mol%) and tris(dibenzylideneacetone) dipalladium(0) (58 mg, 10 mol%) were added and the reaction heated under reflux for 18 h. The mixture was then allowed to cool, water was added and the whole extracted with ethyl acetate (×2). The combined organic extracts were washed with brine (×2), dried, filtered and concentrated *in vacuo*. The crude solid was purified by flash chromatography (10% ethyl acetate in hexanes) to provide compound **35** (34 mg, 17%) as a yellow solid, mp 110–120 °C. ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.74 (d, J = 9.0 Hz, 1H), 7.05 (dd, J = 9.0, 2.0 Hz, 1H), 6.52 (d, J = 2.0 Hz, 1H), 5.35 (br s, 1H), 3.69–3.65 (m, 2H), 3.19 (t, J = 7.0 Hz, 2H), 2.65 (s, 3H), 1.74–1.65 (m, 4H), 1.52–1.43 (m, 4H), 1.00 (t, J = 7.5 Hz, 6H). NH not observed. ^{13}C NMR (100 MHz, $CDCl_3$): δ 155.0, 154.9, 147.1, 128.4, 125.7, 121.5, 119.3, 116.8, 98.9, 91.8, 43.8, 41.5, 31.8, 31.5, 23.9, 20.43, 20.45, 14.05, 14.04. LRMS-ESI (*m*/*z*): 311.2 (M + H)$^+$. HRMS-ESI (*m*/*z*): (M + H)$^+$ calcd for $C_{19}H_{27}N_4^+$, 311.2230; found, 311.2227. RP-HPLC: t_R 11.0 min (Method A).

7-(Benzylamino)-1-(butylamino)-3-methylisoquinoline-4-carbonitrile (36). A mixture of **31** (0.10 g, 0.30 mmol), benzylamine (41 μL, 0.38 mmol) and potassium carbonate (96 mg, 0.69 mmol) in anhydrous *tert*-butanol (10 mL) was degassed under nitrogen. To this mixture 2-dicyclohexylphosphino-2′,4′,6′-triisopropylbiphenyl (30 mg, 20 mol%) and tris(dibenzylideneacetone)dipalladium(0) (29 mg, 10 mol%) were added and the reaction heated under reflux for 17 h. The mixture was then allowed to cool, water was added and the whole extracted with ethyl acetate (×2). The combined organic extracts were washed with brine (×2), dried, filtered and concentrated *in vacuo*. The crude solid was purified by flash chromatography (10% ethyl acetate in hexanes) to provide compound **36** (60 mg, 55%) as a deep red solid, mp 179–181 °C. ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.75 (d, J = 9.0 Hz, 1H), 7.41–7.36 (m, 4H), 7.33–7.29 (m, 1H), 7.09 (dd, J = 9.0, 2.5 Hz, 1H), 6.56 (d, J = 2.5 Hz, 1H), 5.24 (br s, 1H), 4.43 (s, 2H), 3.64–3.61 (m, 2H), 2.64 (s,

3H), 1.69–1.63 (m, 2H), 1.47–1.39 (m, 2H), 0.98 (t, J = 7.5 Hz, 3H). NH not observed. ^{13}C NMR (100 MHz, $CDCl_3$): δ 155.2, 154.8, 146.5, 138.4, 128.9, 128.6, 127.6, 127.5, 125.7, 121.4, 119.1, 116.6, 99.4, 91.7, 48.4, 41.3, 31.6, 23.8, 20.2, 13.9. LRMS-ESI (m/z): 345.2 $(M + H)^+$. HRMS-ESI (m/z): $(M + H)^+$ calcd for $C_{22}H_{25}N_4^+$, 345.2074; found, 345.2070. RP-HPLC: t_R 20.7 min (Method B).

1-(Butylamino)-3-methyl-7-nitroisoquinoline-4-carbonitrile (37). Isoquinoline **4** (0.30 g, 1.3 mmol) was added portion wise to ice cooled concentrated sulfuric acid (6 mL). Once the solid was dissolved potassium nitrate (0.19 g, 1.9 mmol) was added in portions and the reaction mixture stirred at 0 °C for 0.5 h. The reaction mixture was poured onto ice and the solid that separated was filtered, washed with water and recrystallised from ethanol to provide compound **37** (0.27 g, 76%) as a bright orange solid, mp 214–216 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 9.38 (d, J = 2.0 Hz, 1H), 8.99 (br t, J = 5.5 Hz, 1H), 8.47 (dd, J = 9.0, 2.0 Hz, 1H), 7.85 (d, J = 9.0 Hz, 1H), 3.60–3.55 (m, 2H), 2.59 (s, 3H), 1.69–1.62 (m, 2H), 1.43–1.33 (m, 2H), 0.94 (t, J = 7.5 Hz, 3H). ^{13}C NMR (100 MHz, DMSO-d_6): δ 163.1, 157.1, 144.8, 139.6, 125.7, 124.7, 121.1, 117.6, 114.2, 89.2, 40.9, 30.3, 24.3, 19.7, 13.7. LRMS-ESI (m/z): 285.1 $(M + H)^+$. HRMS-ESI (m/z): $(M + H)^+$ calcd for $C_{15}H_{17}N_4O_2^+$, 285.1346; found, 285.1346. RP-HPLC: t_R 20.0 min (Method A).

7-Amino-1-(butylamino)-3-methylisoquinoline-4-carbonitrile (38). A suspension of **37** (1.0 g, 3.5 mmol) and ethanol (40 mL) in the presence of 10% palladium on charcoal (8% w/w, 80 mg) was hydrogenated at atmospheric pressure at room temperature for 0.5 h. The reaction mixture was filtered through a pad of Celite®, the filter cake rinsed with hot ethanol and the solvent was removed *in vacuo*. The crude product was recrystallised from toluene to afford amine **38** (0.73 g, 82%) as a dull mauve solid, mp 180–182 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 7.71 (br t, J = 5.5 Hz, 1H), 7.47 (d, J = 9.0 Hz, 1H), 7.20 (d, J = 2.0 Hz, 1H), 7.16 (dd, J = 9.0, 2.0 Hz, 1H), 5.45 (br s, 2H), 3.53–3.49 (m, 2H), 2.48 (s, 3H), 1.65–1.58 (m, 2H), 1.40–1.31 (m, 2H), 0.92 (t, J = 7.0 Hz, 3H). ^{13}C NMR (100 MHz, DMSO-d_6): δ 155.0, 153.6, 147.4, 126.3, 123.9, 122.0, 119.0, 117.0, 103.3, 89.2, 40.4, 30.7, 23.4, 19.7, 13.8. LRMS-ESI (m/z): 255.1 $(M + H)^+$. HRMS-ESI (m/z): $(M + H)^+$ calcd for $C_{15}H_{19}N_4^+$, 255.1604; found, 255.1605. RP-HPLC: t_R 10.5 min (Method A).

***N*-(1-(Butylamino)-4-cyano-3-methylisoquinolin-7-yl)acetamide (39).** To a solution of amine **38** (50 mg, 0.20 mmol) in anhydrous tetrahydrofuran (5 mL) at 0 °C was added triethylamine (41 μL, 0.30 mmol) and acetyl chloride (21 μL, 0.30 mmol) and the mixture was stirred for 5 min. The reaction mixture was warmed to room temperature and stirred for a further 2 h. The mixture was concentrated *in vacuo*, water was added and the whole extracted with ethyl acetate (×3). The combined organic extracts were washed with brine (×2), dried, filtered and concentrated *in vacuo*. The crude product was purified by flash chromatography (50% ethyl acetate in hexanes) and subsequently recrystallised from toluene to afford acetamido **39** (42 mg, 72%) as off-white needles, mp 240–242 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 10.19 (br s, 1H), 8.45 (d, J = 2.0 Hz, 1H), 8.14 (br t, J = 5.5 Hz, 1H), 7.76 (dd, J = 9.0, 2.0 Hz, 1H), 7.70 (d, J = 9.0 Hz, 1H), 3.57–3.52 (m, 2H), 2.54 (s, 3H), 2.10 (s, 3H), 1.66–1.59 (m, 2H), 1.40–1.31 (m, 2H), 0.92 (t, J = 7.5 Hz, 3H). ^{13}C NMR (100 MHz, DMSO-d_6): δ 168.4, 157.4, 156.0, 137.0, 131.5, 125.8, 123.6, 118.5, 115.5, 113.2, 88.9, 40.6, 30.6, 23.79, 23.78, 19.7, 13.8. LRMS-ESI (m/z): 297.2 $(M + H)^+$. HRMS-ESI (m/z): $(M + H)^+$ calcd for $C_{17}H_{21}N_4O^+$, 297.1710; found, 297.1711. RP-HPLC: t_R 10.7 min (Method A).

1-(1-(Butylamino)-4-cyano-3-methylisoquinolin-7-yl)urea (40). To a solution of amine **38** (50 mg, 0.20 mmol) in acetic acid (5 mL) was added sodium cyanate (25 mg, 0.38 mmol) in water (1 mL) and the mixture allowed to stir at room temperature for 16 h. The reaction was poured onto ice and the solution neutralised with saturated sodium hydrogen carbonate. The solid that separated was filtered, stirred in dichloromethane (5 mL) and subsequently filtered. The crude product was recrystallised from ethanol to afford urea **40** (18 mg, 31%) as a white solid, mp 290 °C (dec.). ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.75 (br s, 1H), 8.14 (d, J = 2.0 Hz, 1H), 8.05 (br t, J = 5.5 Hz, 1H), 7.81 (dd, J = 9.0, 2.0 Hz, 1H), 7.64 (d, J = 9.0 Hz, 1H), 6.03 (br s, 2H), 3.56–3.51 (m, 2H), 2.52 (s, 3H), 1.66–1.59 (m, 2H), 1.41–1.31 (m, 2H), 0.93 (t, J = 7.5 Hz, 3H). ^{13}C NMR (100 MHz, DMSO-d_6): δ 156.5, 155.9, 155.8, 138.7, 130.2, 125.0, 123.5, 118.6, 115.8, 110.9, 88.9, 40.5, 30.6, 23.7, 19.7, 13.8. LRMS-ESI (m/z): 298.2 $(M + H)^+$. HRMS-ESI (m/z): $(M + H)^+$ calcd for $C_{16}H_{20}N_5O^+$, 298.1662; found, 298.1665. RP-HPLC: t_R 9.85 min (Method A).

1-(Butylamino)-7-((4-chlorobenzylidene)amino)-3-methylisoquinoline-4-carbonitrile (41). A suspension of amine **38** (0.10 g, 0.39 mmol) in ethanol (10 mL) was heated to complete dissolution and then allowed to cool to room temperature. To this was added 4-chlorobenzaldehyde (61 mg, 0.43 mmol) and the reaction was stirred for 2 h. Upon reaction completion, as indicated by ^{1}H-NMR, the mixture was cooled on ice and the solid that separated was filtered and washed with cold ethanol. The crude product was recrystallised from toluene to give imine **41** (70 mg, 47%) as a brown solid, mp 177–179 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.77 (s, 1H), 8.30 (br t, J = 5.5 Hz, 1H), 8.24 (d, J = 1.0 Hz, 1H), 8.00 (d, J = 8.5 Hz, 2H), 7.81–7.76 (m, 2H), 7.63 (d, J = 8.5 Hz, 2H), 3.60–3.55 (m, 2H), 2.57 (s, 3H), 1.69–1.61 (m, 2H), 1.43–1.33 (m, 2H), 0.93 (t, J = 7.5 Hz, 3H). ^{13}C NMR (100 MHz, DMSO-d_6): δ 160.3, 158.5, 156.4, 148.9, 136.3, 134.7, 133.7, 130.3, 129.1, 126.0, 124.2, 118.4, 115.8, 115.6, 89.1, 40.6, 30.6, 23.9, 19.7, 13.8. LRMS-ESI (m/z): 377.2 $(M[^{35}Cl] + H)^+$, 379.2 $(M[^{37}Cl] + H)^+$. HRMS-ESI (m/z): $(M[^{35}Cl] + H)^+$ calcd for $C_{22}H_{22}ClN_4^+$, 377.1528; found, 377.1519. RP-HPLC: t_R 10.5 min (Method A).

1-(Butylamino)-7-((4-chlorobenzyl)amino)-3-methylisoquinoline-4-carbonitrile (42). To a suspension of sodium borohydride (60 mg, 1.6 mmol) in ethanol (5 mL) was added imine **41** (60 mg, 0.16 mmol) and the mixture was stirred at room temperature for 16 h. Water was added and the solid that separated was filtered and washed with water. The crude solid was recrystallised in toluene to give compound **42** (34 mg, 56%) as a plum solid, mp 180–182 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 7.76 (br t, J = 5.0 Hz, 1H), 7.48 (d, J = 9.0 Hz, 1H), 7.44 (d, J =

9.0 Hz, 2H), 7.38 (d, J = 9.0 Hz, 2H), 7.24 (dd, J = 9.0, 2.0 Hz, 1H), 7.16 (d, J = 2.0 Hz, 1H), 6.66 (br t, J = 6.0 Hz, 1H) 4.40 (d, J = 6.0 Hz, 2H), 3.55–3.50 (m, 2H), 2.48 (s, 3H), 1.65–1.57 (m, 2H), 1.39–1.30 (m, 2H), 0.92 (t, J = 7.5 Hz, 3H). ^{13}C NMR (100 MHz, DMSO-d_6): δ 155.0, 153.8, 147.2, 138.6, 131.4, 129.5, 128.2, 126.6, 123.9, 122.2, 118.9, 116.8, 99.8, 89.2, 45.7, 40.4, 30.8, 23.5, 19.8, 13.8. LRMS-ESI (m/z): 379.2 (M[^{35}Cl] + H)$^+$, 381.2 (M[^{37}Cl] + H)$^+$. HRMS-ESI (m/z): (M[^{35}Cl] + H)$^+$ calcd for $C_{22}H_{24}ClN_4^+$, 379.1684; found, 379.1685. RP-HPLC: t_R 16.0 min (Method A).

1-(Butylamino)-7-iodo-3-methylisoquinoline-4-carbonitrile (43). To a suspension of amine **38** (0.10 g, 0.39 mmol) in concentrated hydrochloric acid (4 mL), at −5° C, was added sodium nitrite (54 mg, 0.79 mmol) in water (2.5 mL) dropwise over 5 min and the reaction mixture was stirred for 1 h. The resulting solution was added dropwise to a solution of potassium iodide (1.6 g, 9.8 mmol) in water (8 mL) and the reaction mixture stirred at −5° C for 0.25 h and for a further 4 h at room temperature. The reaction mixture was diluted with water and extracted with ethyl acetate (×3). The combined organic extracts were washed with 2.5 M sodium hydroxide (×2), 10% sodium thiosulfate (×2), 2.5 M sodium hydroxide, water, brine, dried, filtered and concentrated *in vacuo* to provide compound **43** (0.10 g, 70%) as a brown solid, mp 204–208 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.78 (d, J = 1.5 Hz, 1H), 8.37 (br t, J = 5.0 Hz, 1H), 8.05 (dd, J = 9.0, 1.5 Hz, 1H), 7.51 (d, J = 9.0 Hz, 1H), 3.57–3.52 (m, 2H), 2.54 (s, 3H), 1.67–1.59 (m, 2H), 1.42–1.32 (m, 2H), 0.93 (t, J = 7.5 Hz, 3H). ^{13}C NMR (100 MHz, DMSO-d_6): δ 159.4, 155.0, 140.0, 134.4, 132.1, 124.9, 117.9, 116.7, 91.3, 88.9, 40.7, 30.4, 24.0, 19.8, 13.8. LRMS-ESI (m/z): 366.0 (M + H)$^+$. RP-HPLC: t_R 22.3 min (Method B).

1-(Butylamino)-7-ethoxy-3-methylisoquinoline-4-carbonitrile (44). To a suspension of **43** (0.10 g, 0.27 mmol), caesium carbonate (0.18 g, 0.55 mmol) and 1,10-phenanthroline (9.9 mg, 20 mol%) in ethanol (4.4 mL) was added copper(I) iodide (5.2 mg, 10 mol%) and the mixture heated to 110 °C in a pressure tube for 18 h. The reaction mixture was allowed to cool, filtered through a silica pad and solvent removed *in vacuo* to give a brown solid. The solid was purified by flash chromatography (10% ethyl acetate in hexanes) to provide compound **44** (34 mg, 44%) as a white solid, mp 150–161 °C. ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.86 (d, J = 9.0 Hz, 1H), 7.34 (dd, J = 9.0, 2.5 Hz, 1H), 6.98 (d, J = 2.5 Hz, 1H), 5.47 (br s, 1H), 4.14 (q, J = 7.0 Hz, 2H), 3.69–3.65 (m, 2H), 2.67 (s, 3H), 1.74–1.68 (m, 2H), 1.51–1.43 (m, 5H), 1.00 (t, J = 7.5 Hz, 3H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 157.4, 156.9, 155.2, 130.7, 126.2, 122.1, 118.8, 116.1, 103.0, 91.6, 64.1, 41.4, 31.6, 23.9, 20.3, 14.8, 13.9. LRMS-ESI (m/z): 284.1 (M + H)$^+$. HRMS-ESI (m/z): (M + H)$^+$ calcd for $C_{17}H_{22}N_3O^+$, 284.1757; found, 284.1760. RP-HPLC: t_R 14.0 min (Method A).

7-Bromo-1-(butylamino)-3-methyl-5-nitroisoquinoline-4-carbonitrile (45). Compound **31** (0.40 g, 1.3 mmol) was added portion wise to ice cold concentrated sulfuric acid (8 mL). Once the solid was dissolved, potassium nitrate (0.19 g, 1.9 mmol) was added in small portions and the reaction mixture stirred at 0 °C for 1 h. The reaction mixture was poured on ice and the solid that separated was filtered, washed with water and recrystallised from methanol to provide compound **45** (0.36 g, 79%) as a deep red solid, mp 211–213 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.91 (d, J = 2.0 Hz, 1H), 8.77 (br t, J = 5.5 Hz, 1H), 8.54 (d, J = 2.0 Hz, 1H), 3.60–3.55 (m, 2H), 2.58 (s, 3H), 1.68–1.61 (m, 2H), 1.42–1.33 (m, 2H), 0.93 (t, J = 7.5 Hz, 3H). ^{13}C NMR (100 MHz, DMSO-d_6): δ 163.8, 155.2, 145.9, 130.3, 129.7, 124.4, 117.6, 117.0, 115.7, 83.6, 41.0, 30.2, 24.6, 19.7, 13.7. LRMS-ESI (m/z): 363.1 (M[^{79}Br] + H)$^+$, 365.1 (M[^{81}Br] + H)$^+$. HRMS-ESI (m/z): (M[^{79}Br] + H)$^+$ calcd for $C_{15}H_{16}BrN_4O_2^+$, 363.0451; found, 363.0453. RP-HPLC: t_R 20.7 min (Method A).

1-(Butylamino)-3-methyl-5-nitro-7-phenylisoquinoline-4-carbonitrile (46). Compound **45** (0.20 g, 0.55 mmol) was coupled to phenylboronic acid (0.10 g, 0.83 mmol) according to General procedure D. The crude solid was purified by flash chromatography (10% ethyl acetate in hexanes) to provide compound **46** (54 mg, 27%) as a yellow solid, mp 240–245 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.92–8.90 (m, 2H), 8.60 (d, J = 1.5 Hz, 1H), 7.94–7.92 (m, 2H), 7.59–7.55 (m, 2H), 7.50–7.46 (m, 1H), 3.66–3.61 (m, 2H), 2.61 (s, 3H), 1.72–1.64 (m, 2H), 1.45–1.35 (m, 2H), 0.95 (t, J = 7.5 Hz, 3H). ^{13}C NMR (100 MHz, DMSO-d_6): δ 163.2, 156.2, 146.1, 137.2, 136.8, 129.1, 128.7, 127.1, 125.4, 124.8, 124.4, 116.9, 116.1, 83.6, 40.9, 30.4, 24.5, 19.7, 13.8. LRMS-ESI (m/z): 361.1 (M + H)$^+$. HRMS-ESI (m/z): (M + H)$^+$ calcd for $C_{21}H_{21}N_4O_2^+$, 361.1659; found, 361.1664. RP-HPLC: t_R 21.3 min (Method B).

5-Amino-1-(butylamino)-3-methylisoquinoline-4-carbonitrile (47). A suspension of **45** (0.52 g, 1.4 mmol), triethylamine (0.20 mL, 1.4 mmol) and ethanol (20 mL) in the presence of 10% palladium on charcoal (8% w/w, 40 mg) was hydrogenated at atmospheric pressure at room temperature for 2 h. The reaction mixture was filtered through a pad of Celite®, the filter cake was rinsed with hot ethanol and the solvent was removed *in vacuo*. The crude residue was dissolved in ethyl acetate, washed with water (×2), brine (×2), dried, filtered and concentrated *in vacuo*. The crude mixture was recrystallised from petroleum ether (bp 80–110 °C)/toluene to afford amine **47** (0.31 g, 85%) as a maroon solid, mp 151–154 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.01 (br t, J = 5.0 Hz, 1H), 7.50 (d, J = 8.0 Hz, 1H), 7.24 (dd app t, J = 8.0 Hz, 1H), 6.95 (d, J = 8.0 Hz, 1H), 5.65 (s, 2H), 3.55–3.50 (m, 2H), 2.54 (s, 3H), 1.65–1.57 (m, 2H), 1.39–1.30 (m, 2H), 0.92 (t, J = 7.0 Hz, 3H). ^{13}C NMR (100 MHz, DMSO-d_6): δ 159.0, 156.7, 143.0, 126.8, 122.1, 122.0, 116.6, 115.8, 111.7, 85.0, 40.6, 30.7, 24.2, 19.8, 13.8. LRMS-ESI (m/z): 255.1 (M + H)$^+$. HRMS-ESI (m/z): (M + H)$^+$ calcd for $C_{15}H_{19}N_4^+$, 255.1604; found, 255.1604. RP-HPLC: t_R 10.4 min (Method A).

***N*-(1-(Butylamino)-4-cyano-3-methylisoquinolin-5-yl)acetamide (48).** To a solution of amine **47** (50 mg, 0.20 mmol) in anhydrous tetrahydrofuran (5 mL) at 0 °C was added triethylamine (55 μL, 0.40 mmol) and acetyl chloride (28 μL, 0.40 mmol) and the mixture was stirred for 5 min. The reaction mixture was warmed to room temperature and stirred for a further 4 h. The mixture was concentrated *in vacuo*, water

was added and the whole extracted with ethyl acetate (×3). The combined organic extracts were washed with brine (×2), dried, filtered and concentrated *in vacuo*. The crude product was recrystallised from ethanol to afford acetamido **48** (45 mg, 77%) as a pink solid, mp 245–248 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 9.88 (s, 1H), 8.27 (br t, J = 5.5 Hz, 1H), 8.22 (dd, J = 8.0, 1.0 Hz, 1H), 7.53–7.45 (m, 2H), 3.59–3.54 (m, 2H), 2.56 (s, 3H), 2.07 (s, 3H), 1.67–1.60 (m, 2H), 1.42–1.32 (m, 2H), 0.93 (t, J = 7.0 Hz, 3H). ^{13}C NMR (100 MHz, DMSO-d_6): δ 169.7, 160.9, 156.3, 132.7, 131.9, 131.6, 125.7, 122.2, 119.9, 116.4, 86.5, 40.6, 30.6, 24.3, 22.9, 19.7, 13.8. LRMS-ESI (m/z): 297.2 (M + H)$^+$. HRMS-ESI (m/z): (M + H)$^+$ calcd for $C_{17}H_{21}N_4O^+$, 297.1710; found, 297.1710. RP-HPLC: t_R 8.58 min (Method A).

1-(Butylamino)-5-((4-chlorobenzylidene)amino)-3-methylisoquinoline-4-carbonitrile (49). A suspension of amine **47** (50 mg, 0.20 mmol) in ethanol (5 mL) was heated to complete dissolution and then allowed to cool to room temperature. To this was added 4-chlorobenzaldehyde (41 mg, 0.30 mmol) and the reaction refluxed for 24 h. Upon reaction completion, as indicated by ^{1}H-NMR, the mixture was cooled on ice and the solid that separated was filtered and washed with cold ethanol. The crude product was recrystallised from toluene/petroleum ether (bp 80–110 °C) to give imine **49** (29 mg, 39%) as a brown solid, mp 176–177.5 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.63 (s, 1H), 8.23 (br t, J = 5.5 Hz, 1H), 8.18 (dd, J = 8.0, 1.0 Hz, 1H), 8.10 (d, J = 8.5 Hz, 2H), 7.62 (d, J = 8.5 Hz, 2H), 7.54 (dd app t, J = 8.0 Hz, 1H), 7.40 (dd, J = 8.0, 1.0 Hz, 1H), 3.60–3.55 (m, 2H), 2.59 (s, 3H), 1.69–1.61 (m, 2H), 1.43–1.33 (m, 2H), 0.94 (t, J = 7.5 Hz, 3H). ^{13}C NMR (100 MHz, DMSO-d_6): δ 160.9, 159.7, 156.1, 146.1, 136.3, 134.6, 131.3, 130.1, 128.8, 126.4, 121.1, 120.5, 119.4, 116.1, 86.9, 40.6, 30.6, 24.4, 19.7, 13.8. LRMS-ESI (m/z): 377.3 (M[^{35}Cl] + H)$^+$, 379.2 (M[^{37}Cl] + H)$^+$. HRMS-ESI (m/z): (M[^{35}Cl] + H)$^+$ calcd for $C_{22}H_{22}ClN_4^+$, 377.1528; found, 377.1533. RP-HPLC: t_R 20.8 min (Method B).

1-(Butylamino)-5-((4-chlorobenzyl)amino)-3-methylisoquinoline-4-carbonitrile (50). To a suspension of sodium borohydride (0.10 g, 2.7 mmol) in ethanol (10 mL) was added imine **49** (0.10 g, 0.27 mmol) and the mixture was stirred at room temperature for 16 h. Water was added and the solid that separated was filtered and washed with water. Purification by flash chromatography (15% → 100% ethyl acetate in hexanes) and subsequent recrystallisation from petroleum ether (bp 80–110 °C)/toluene provided compound **50** (34 mg, 34%) as a pale green solid, mp 142–144 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.05 (br t, J = 6.0 Hz, 1H), 7.56 (d, J = 8.0 Hz, 1H), 7.49 (d, J = 9.0 Hz, 2H), 7.40 (d, J = 9.0 Hz, 2H), 7.28 (dd app t, J = 8.0 Hz, 1H), 6.76 (d, J = 8.0 Hz, 1H), 6.24 (br t, J = 5.5 Hz, 1H), 4.43 (d, J = 5.5 Hz, 2H), 3.57–3.52 (m, 2H), 2.58 (s, 3H), 1.65–1.58 (m, 2H), 1.40–1.31 (m, 2H), 0.92 (t, J = 7.5 Hz, 3H). ^{13}C NMR (100 MHz, DMSO-d_6): δ 159.5, 156.5, 142.2, 138.0, 131.4, 129.2, 128.3, 126.7, 123.8, 121.6, 116.3, 112.0, 111.6, 85.1, 46.8, 40.6, 30.7, 24.3, 19.7, 13.8. LRMS-ESI (m/z): 379.3 (M[^{35}Cl] + H)$^+$, 381.0 (M[^{37}Cl] + H)$^+$. HRMS-ESI (m/z): (M[^{35}Cl] + H)$^+$ calcd for $C_{22}H_{24}ClN_4^+$, 379.1684; found, 379.1685. RP-HPLC: t_R 23.0 min (Method B).

Malstat assays for growth inhibition

P. falciparum 3D7 and W2mef parasites were cultured according to the procedure described by Trager *et al.*[53] in RPMI-HEPES media supplemented with L-glutamine (Sigma) and Albumax II (Invitrogen). 100 μL of *P. falciparum* ring-stage cultures were seeded into 96-well microplates at 0.3% parasitaemia and 2% haematocrit. Compounds were serially diluted in DMSO at 500× working concentration and were added to cultures with a final concentration of 0.2% DMSO. The growth assays were performed in triplicate for 1.5 cell cycles (37 °C, 72 h). The cultures were then lysed by a freeze–thaw cycle and 30 μL was mixed with 75 μL Malstat reagent (0.1 M Tris pH 8.5), 0.2 g mL^{-1} lactic acid, 0.2% v/v Triton X-100 and 1 mg mL^{-1} acetylpyridine adenine dinucleotide [Sigma], 0.01 mg ml^{-1} phenozine ethosulfate [Sigma] and 0.2 mg mL^{-1} nitro blue tetrazolium [Sigma]. Once the no drug control wells had developed a purple color the absorbance was measured at 650 nm in a spectrophotometer. Absorbance values were plotted in Prism 6 (GraphPad) and normalised against untreated and uninfected samples to generate drug curves and IC_{50} values.

Expression of recombinant *Pf*PKA

Synthetic cDNA encoding the *Plasmodium falciparum* 3D7 PKA catalytic subunit (CAA11945) was cloned into pET-28b based expression vector. Two different constructs were trialled consisting of residues 1–342 and 14–342. Recombinant protein was expressed in *E. coli* BL21(DE3) cells grown for 4 hours at 37 °C in Luria Broth containing 34 μM Kanamycin before being induced for 4–22 hours at 18, 22 or 37 °C with 0.1 mM Isopropyl thiogalactopyranoside. Cells were harvested and resuspended in buffer A (20 mM HEPES pH 7.5, 250 mM NaCl, 1 mM TCEP) and lysed using sonication and 1 mg mL^{-1} Lysozyme and centrifuged at 15 000*g* for 30 minutes. Samples of the re-solubilised pellet and supernatant where run on SDS-PAGE gels.

In vitro phosphorylation assays

Nunc MaxiSorp 96 well ELISA plates were coated with 1 μg ml^{-1} of the GST-*Pf*AMA1 substrate. Phosphorylation reactions were performed in the wells of the plate in kinase activity buffer (20 mM Tris, 20 mM $MgCl_2$, 2 mM $MnCl_2$, 1 mM DTT, Roche PhosSTOP) with 2% parasite extract, 100 μM ATP and 2 μM cAMP and 10 μM H89 as indicated (37 °C, 30 min). Inhibitors were titrated at a final concentration of 0.1% DMSO. Following the reaction the wells were washed with TBS and probed with a rabbit anti phospho-*Pf*AMA1$_{S610}$, and then washed again with TBS and probed with goat anti rabbit-HRP (Abcam). To develop the assay ABTS was added to the wells for 30 min and the reaction was stopped with 1% SDS before reading at 405 nm. Absorbance values were plotted in Prism 6 (GraphPad).

Luciferase assays for egress and invasion inhibition

P. falciparum 3D7 parasites transfected with an exported NanoLuc fusion protein[39] were cultured as above. The com-

pounds were serially diluted as above and added to synchronised late-stage parasite cultures (46–48 h post invasion) and were seeded into 96-well microplates at 5% parasitaemia and 2% haematocrit. Compounds were titrated in triplicate and incubated over egress and invasion (37 °C, 4 h). Samples of culture supernatant were collected to determine the amount of luciferase released during egress. Samples were treated with 5% sorbitol in water to lyse any unruptured schzionts and were then washed in 3× in 2 volumes of culture medium to wash out the PKA inhibitors. The parasites were then returned to culture (37 °C, 24 h) and samples were collected again to determine the amount of luciferase carried over during invasion. From the egress media and the 24 h old cultures, a small aliquot was removed (5–10 μL) and added to 0.6× sample volume PBS, 0.4× sample volume Luciferase Cell Culture Lysis 5X Reagent (Promega) and 0.01× sample volume NanoGlo Luciferase Assay Substrate (Promega). Relative light units (RLU) emitted from the reaction were measured in a FLUOstar Omega Luminometer (BMG Labtech) with the gain reduced by 10% for the sample with the highest signal to prevent saturation. Relative light units were plotted in Prism 6 (GraphPad) and normalised against untreated and uninfected samples to generate drug curves and IC_{50} values.

Video microscopy

P. falciparum W2mef strain asexual blood stage parasites were grown to the late schizont stage. They were diluted from 4% to 0.16% hematocrit in RPMI media to which the *Pf*PKA inhibitors had been added and were allow to settle on the bottom of a 35 mm Fluorodish (World Precision Instruments). The dishes were mounted in a humidified gas chamber (90% N_2, 1% O_2, and 5% CO_2) at 37 °C on a Zeiss AxioObserver Z1 fluorescence microscope and time-lapse videos were recorded with an AxioCam MRm camera usually at 4 frames per second.

Human kinase screen

Compound 25 was tested at 10 mM against a panel of available human kinases with homologs in *P. falciparum* by MRC PPU International Centre for Kinase Profiling (University of Dundee, United Kingdom). The principal method utilised is a radioactive filter binding assay using ^{33}P ATP.[54,55] The enzyme–substrate mixture is incubated for 5 min at rt followed by the addition of ^{33}P ATP to begin the reaction. The assay is halted by orthophosphoric acid and the components harvested onto a P81 filter plate then air-dried. Scintillation counting is undertaken using a Topcount NXT. A mean percentage activity with standard deviation for all duplicates is calculated.

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