

Epigenetic therapeutics in malaria:

A chemical biological approach towards the validation of histone lysine methyltransferase inhibition in *Plasmodium falciparum*

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PhD Thesis

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March 2018

Declaration of Originality

I confirm that the work presented within this thesis is entirely my own, conducted under the supervision of Dr Matthew J. Fuchter, at the Department of Chemistry, Imperial College London, unless otherwise stated. All work performed by others has been acknowledged within the text and referenced where appropriate.

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Abstract

A growing threat from drug resistance means there is an urgent need for new therapeutics and novel mechanisms of action for antimalarial drug discovery. Epigenetic mechanisms, including histone methylation, are vital throughout the *Plasmodium* lifecycle, and could provide exciting new targets. BIX-01294 and a series of diaminoquinazolines are putative *Plasmodium* histone lysine methyltransferase inhibitors, with exciting antimalarial properties, although robust evidence for their molecular targets is lacking. To this end, a well-developed SAR for this series allowed for the development of small-molecule photo-crosslinkable probes to investigate the targets. These probes effectively label *Plasmodium falciparum* lysates and show similarities with the target profiles of BIX-01294 and the diaminoquinazoline series. Initial pull-down proteomics experiments with two different probes identified 45 common proteins from different classes, many of which are essential, highlighting the suitability of the developed probes as valuable tools for target identification in *Plasmodium falciparum*.

Acknowledgements

First and foremost, I would like to thank my supervisor Dr Matt Fuchter, for giving me the opportunity to work on this project and his continued support throughout. I have thoroughly enjoyed my PhD and have been lucky to carry out such interesting and varied research. I would like to thank Sandeep for helping so much when I first started, and Ainoa for helping develop the project and for her support whilst I wrote this thesis. Her help and support has been invaluable. I would also like to thank the whole Fuchter group, who have been helpful, supportive, and a lot of fun to be around, and especially Hannah, Luiza, Katie and Melis, who have kept me sane and made the last three years so enjoyable.

A special thanks to the laboratory of Artur Scherf at the Pasteur Institute in Paris, who have collaborated heavily on this project throughout. Particular thanks to Patty, who provided invaluable time, materials, data and advice. I would also like to thank Professor Jake Baum and the members of his group, who allowed me space in their life sciences lab and helped me learn the biological techniques needed for this project, and Professor Ed Tate and his group for their help and support with the proteomics, and for gifting the valuable capture reagents used in this work.

Finally, I would like to thank my mum for her continuous support, and David, without whom I could not have done this.

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Abbreviations

Abbreviation	Meaning
ABPP	Activity-based protein profiling
Ac	Acetyl
ACT	Artemisinin-based combination therapies
AzT	TAMRA-azide
AzTB	Azide-TAMRA-biotin
BSA	Bovine serum albumin
Boc	<i>tert</i> -butoxycarbonyl protecting group
DCM	Dichloromethane
DIPEA	<i>N,N</i> -Diisopropylethylamine
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EI	Electron Impact
ESI	Electrospray mass ionisation
FDA	Food and Drug Administration
FDR	False discovery rate
GO	Gene Ontology
H(K)MT	Histone (lysine) methyltransferase
HAT	Histone acetyl transferase
HDAC	Histone deacetylase
HDM	Histone demethylase
HEPES	4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC	High-performance liquid chromatography
HRMS	High resolution mass spectrometry
IC ₅₀	Half maximal inhibitory concentration
iRBC	Infected red blood cell
LC-MS	Liquid chromatography–mass spectrometry
LFQ	Label-free quantification
Me	Methyl

MS	Mass spectrometry
NMR	Nuclear magnetic resonance
PANTHER	Protein ANALysis THrough Evolutionary Relationships
PBS	Phosphate-buffered saline
<i>Pf</i>	<i>Plasmodium falciparum</i>
<i>PfEMP1</i>	<i>P. falciparum</i> Erythrocyte Membrane Protein-1
PTM	Post-translational modification
RBC	Red blood cell
RNA	Ribonucleic acid
SAM	S-adenosyl methionine
SAR	Structure-activity relationship
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SET	Su (var)3-9, E nhancer-of-zeste and T rithorax
TBTA	Tris((1-benzyl-1 <i>H</i> -1,2,3-triazol-4-yl)-methyl)-amine
TCEP	Tris(2-carboxyethyl)-phosphine
TFA	Trifluoroacetic acid
THF	Tetrahydrofuran
TPSA	Topological polar surface area
UV	Ultra-violet
WHO	World Health Organisation

1. Introduction

1.1. Malaria

Malaria remains one of the deadliest diseases in the developing world. In 2015 there were 212 million cases worldwide, claiming 429 000 lives, mostly from children under 5 years old. The disease is particularly prevalent in Sub-Saharan Africa where in 2015 over 90% of malaria deaths occurred.¹ The large burden of malaria, as well as the many challenges involved in the control and treatment of this disease, mean there remains an urgent and a vital need for novel research and development.

1.1.1. Malaria Lifecycle

Malaria is caused by *Plasmodium* parasites. There are five species known to infect humans: *Plasmodium falciparum*, *P. vivax*, *P. ovale*, *P. malariae* and *P. knowlesi*.² *Plasmodium falciparum* is the biggest cause of mortality. The *Plasmodium* parasite is transmitted to humans by female *Anopheles* mosquitoes, with the complex life-cycle involving the two hosts, as shown in Figure 1.

When an infected mosquito takes a blood-meal, the parasite is injected from the salivary glands of the mosquito into the human host's bloodstream as sporozoites. These are transported to the liver and infect liver cells where they replicate and mature into schizonts. In *P. vivax* and *P. ovale* the parasite can persist at a dormant stage in the liver as hypnozoites causing a relapse weeks, months or even years later. After this initial liver stage, the schizonts rupture to release merozoites into the bloodstream, which then infect red blood cells. The parasite develops into trophozoites, which develop into schizonts, which then rupture and release more merozoites, repeating the 48-hour asexual erythrocytic cycle (see Figure 1B).³

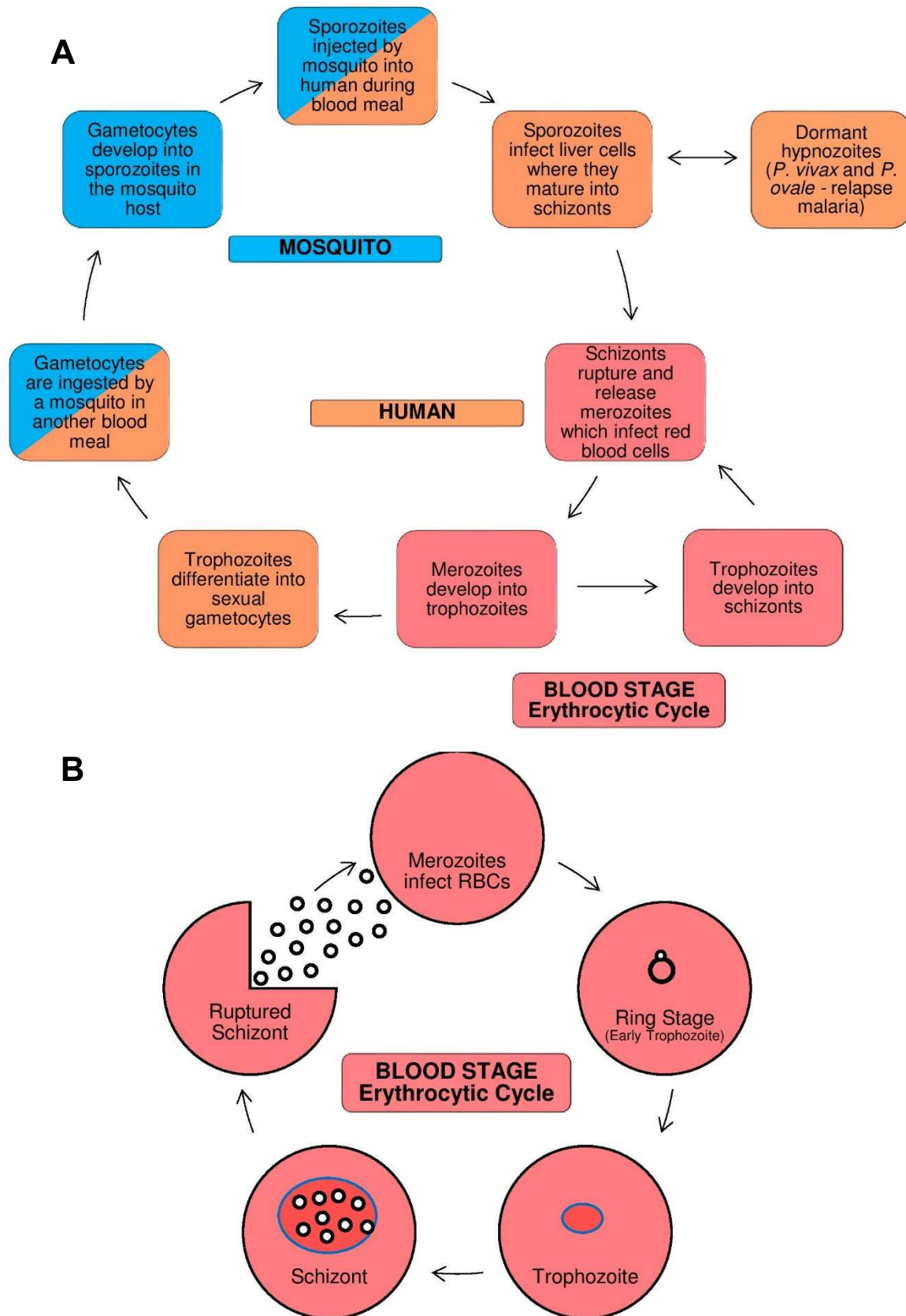


Figure 1: A) Two-host lifecycle of *Plasmodium* involving the mosquito and human liver and blood stages; B) Erythrocytic 48-hour asexual blood cycle of *Plasmodium falciparum*.

Some trophozoites differentiate into sexual gametocytes (macro- and micro-gametocytes), which are ingested by the mosquito. In the mosquito, the male and female gametocytes fertilize each other to produce a zygote that will eventually develop into sporozoites and restart the lifecycle.³

The pathogenesis of malaria is caused by the asexual erythrocytic stage of the *Plasmodium* lifecycle after the infection of the red blood cells by merozoites (Figure 1B). Symptoms of malaria include fever, anaemia, headaches, chills and nausea. When schizonts rupture a number of toxins are released along with merozoites, leading to an immune response that causes the release of inflammatory mediators, and triggering the above mentioned symptoms of malaria.⁴ In addition, late-stage forms of *P. falciparum* (late-stage trophozoites and schizonts) can cause cytoadherence – the infected red blood cells (iRBCs) adhere to the vascular endothelium (the lining of the circulatory system). This prevents the parasite from being cleared by the spleen, allowing it to start another erythrocytic cycle,⁵ and can also cause severe forms of malaria from including severe anaemia and organ failure from the blockage of blood vessels.⁶ When this happens in the brain, severe cerebral malaria can occur, with blocked blood vessels leading to neurological damage, or death.⁷ These complications mean *P. falciparum* accounted for 99% of deaths from malaria in 2015.¹

1.1.2. Current Treatments and Challenges

The first treatment for malaria was quinine (Figure 2), an alkaloid natural product which was first used in the 1600s.⁸ Several analogues of quinine have been developed, including chloroquine (Figure 2) which was first introduced for clinical use in 1947. Chloroquine became the primary treatment for malaria, however by the end of the 1980's there was widespread chloroquine resistance.⁹ Although the mechanism of action of these drugs is not fully understood, they are believed to kill the parasite by preventing the detoxification of a toxic by-product of red blood cell invasion.¹⁰ It has been found that chloroquine accumulates in

parasites to a much lesser extent in resistant strains of parasite than in susceptible ones,¹¹ which is linked to point-mutations in a chloroquine transporter protein (*PfCRT*) and is believed to confer resistance.¹²⁻¹³

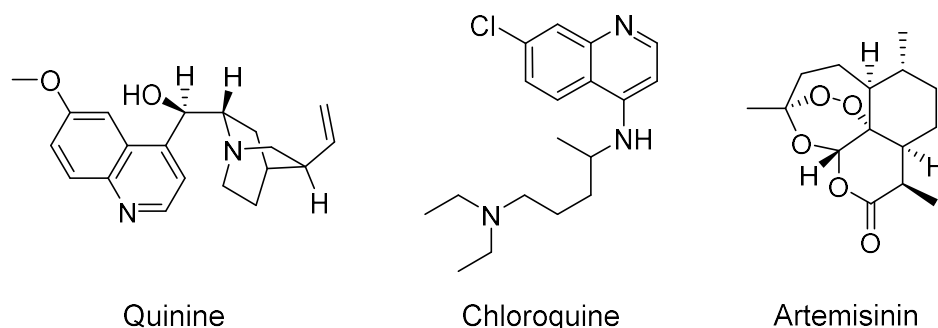


Figure 2: Common antimalarials

Following this there was an urgent need for new drugs, which led to the development of derivatives of artemisinin, shown in Figure 2. Artemisinin was first discovered from the plant *Artemisia annua* by Youyou Tu in China, who won the 2015 Nobel Prize for Physiology or Medicine for the discovery.¹⁴ The reduction of artemisinin to dihydroartemisinin increased the potency of the compound, and allowed for further derivatives via the elaboration of the hydroxyl group, including the methyl derivative artemether.¹⁴⁻¹⁵ Artemisinin derivatives are extremely active against all erythrocytic blood stages, and also reduce transmission by targeting gametocytes.¹⁶ The current recommended treatment for uncomplicated malaria is artemisinin-based combination therapy (ACT), which combines an artemisinin derivative with a second drug such as piperazine (a derivative of chloroquine).¹⁷

There are several reasons why artemisinin derivatives are used in combination with other drugs such as chloroquine, rather than as stand-alone treatments. Firstly, it reduces the risk of resistance, since mutations would have to lead to resistance to both drugs for the parasites to survive.¹⁸ Secondly, artemisinin derivatives are metabolised quickly in the body and so by combination with a drug that has a longer half-life, treatment times can be reduced, and the chance to clear the parasite completely increases.¹⁹

However, artemisinin resistance is now emerging. It was first observed in South-East Asia after concerns of a reduction in the efficacy of ACTs.²⁰ Resistance has now spread within this region,²¹ and has recently been observed in Africa.²² Resistance to partner drugs is also known and so ACTs are now also under serious threat of becoming ineffective from drug resistance world-wide.²³

In addition, ACTs are also ineffective against the hypnozoites that cause relapse malaria,¹⁰ and with the only treatment currently available being unsuitable for mass administration, there is a real need for new ways of tackling this. Hence there is an urgent need for the development of new treatments.

In Sub-Saharan Africa in particular, there are also many other major challenges including a lack of medical equipment and expertise, parts of the population being very difficult to reach, over-prescription of some drugs which can speed up resistance, high costs and the marketing of cheaper fake drugs, amongst other things. Malaria control and treatment is therefore an ongoing challenge.

1.1.3. Future of Malaria Treatment

The Medicines for Malaria Venture (MMV) have a global portfolio of candidates in the clinical pipeline, from lead optimisation through clinical trials up to approval for clinical use. Therapeutics in development include a number of novel treatments including drugs for treating severe malaria, targeting transmission, and even long-lasting seasonal prevention.²⁴ However the majority of new treatments that have made it through clinical trials and been approved are still ACTs, which underlines further the need for new alternatives.

The WHO has set out ambitious plans towards the eradication of malaria, including reducing its burden by 90% by 2030.²⁵ Innovation in both control and treatment of malaria, as well as improved access to these measures are all vital components to reach these goals.

To eradicate malaria, a number of strategies will be required. These will include continued work towards the development of malaria vaccines,²⁶ vector control programmes,²⁷ as well as vital work in the countries themselves to increase access to and awareness of both existing and new prevention and treatment options. With the spread of drug resistance threatening current treatments, new therapeutics to treat malaria will be a vital part of this strategy. Both novel drugs and novel targets may open up new avenues for drug development.

A relatively unexplored avenue for antimalarial drug discovery is targeting epigenetic mechanisms. These processes are vital for parasite survival throughout its life cycle,²⁸⁻³⁰ and so could provide exciting novel drug targets for novel therapeutics.

1.2. Epigenetics and Malaria

Epigenetics is the altering of gene expression without altering the genetic code. Genetic information in the cell is encoded by DNA, with different genes encoded by different regions of DNA. However not all genes are active simultaneously, and different genes are active in different cell types, and at different points during the cell cycle, leading to the complexity of life. This is controlled by epigenetic mechanisms – mechanisms that alter gene expression, without alteration to the underlying genetic code.

1.2.1. Chromatin and Transcriptional Control

Chromatin is the complex of proteins and DNA that forms chromosomes in eukaryotic cells. The basic structural unit of chromatin is the nucleosome, shown in Figure 3. The nucleosome consists of the DNA coiled around a protein core formed by an octamer of proteins known as histones. There are four pairs of histones in the octamer: H2A, H2B, H3 and H4, and together they consist of a structured core and protruding N-terminal ‘tails’.

The local structure of chromatin around a given gene determines whether this gene is 'switched on' or 'switched off'. Thus, transcriptionally active chromatin, or euchromatin, has an open, loose structure that makes genetic information accessible for transcription. Conversely, transcriptionally inactive chromatin, or heterochromatin, has a rigid, tightly-packed structure that hampers the transcription process.

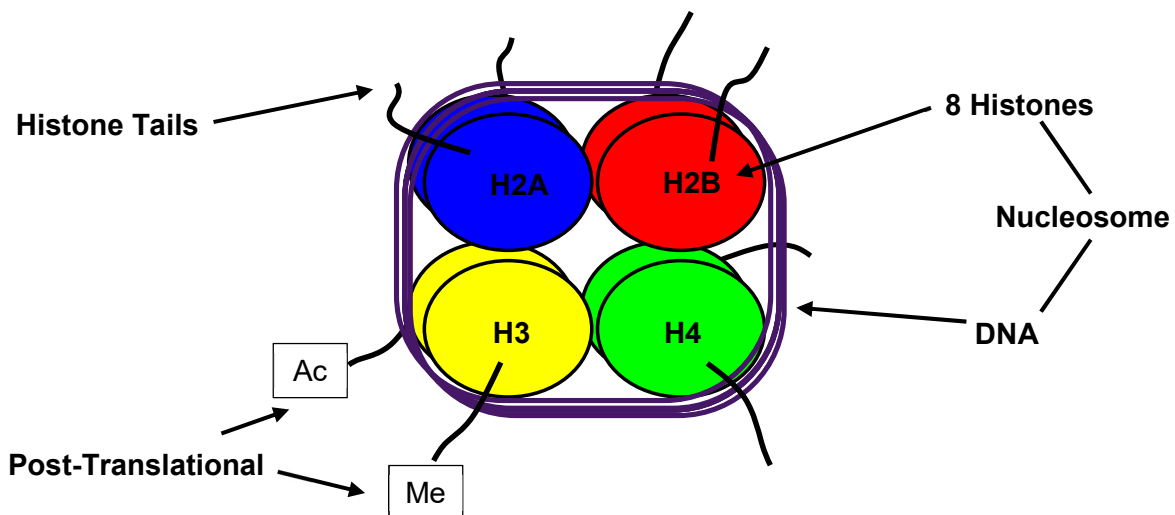


Figure 3: The nucleosome is the basic structural unit of chromatin. It is formed from DNA coiled around an octamer of proteins known as histones. There are four pairs of histones in the octamer: H2A, H2B, H3 and H4, and together they consist of a structured core and protruding N-terminal 'tails', where a number of post-translational modifications are possible.

1.2.2. Transcriptional Control in *Plasmodium*

As mentioned before, the *Plasmodium* parasite has a very complex two-host lifecycle that requires high levels of transcriptional regulation and control for parasite survival. In order to assess in depth the importance of this regulation in the asexual erythrocytic cycle, Bozdech *et al.* analysed the transcriptome of the parasite, using a DNA microarray to examine expression profiles at 1-hour intervals over the 48-hour asexual erythrocytic cycle.³¹ They found that while the majority of genes were activated in this cycle, 75% of them showed a single peak of expression in the cycle. As shown in Figure 4, the phaseogram of the transcriptome shows a continuous cascade of expression, with common expression profiles between related genes, and a close link between the timing of expression and cellular

function. In the ring and early trophozoite stages, genes relating to transcription and translation machinery, and metabolic pathways are at a maximum. Later in the cycle in the schizont and early ring stages, genes related to specific parasite function are expressed, particularly those that facilitate host-cell invasion.³¹

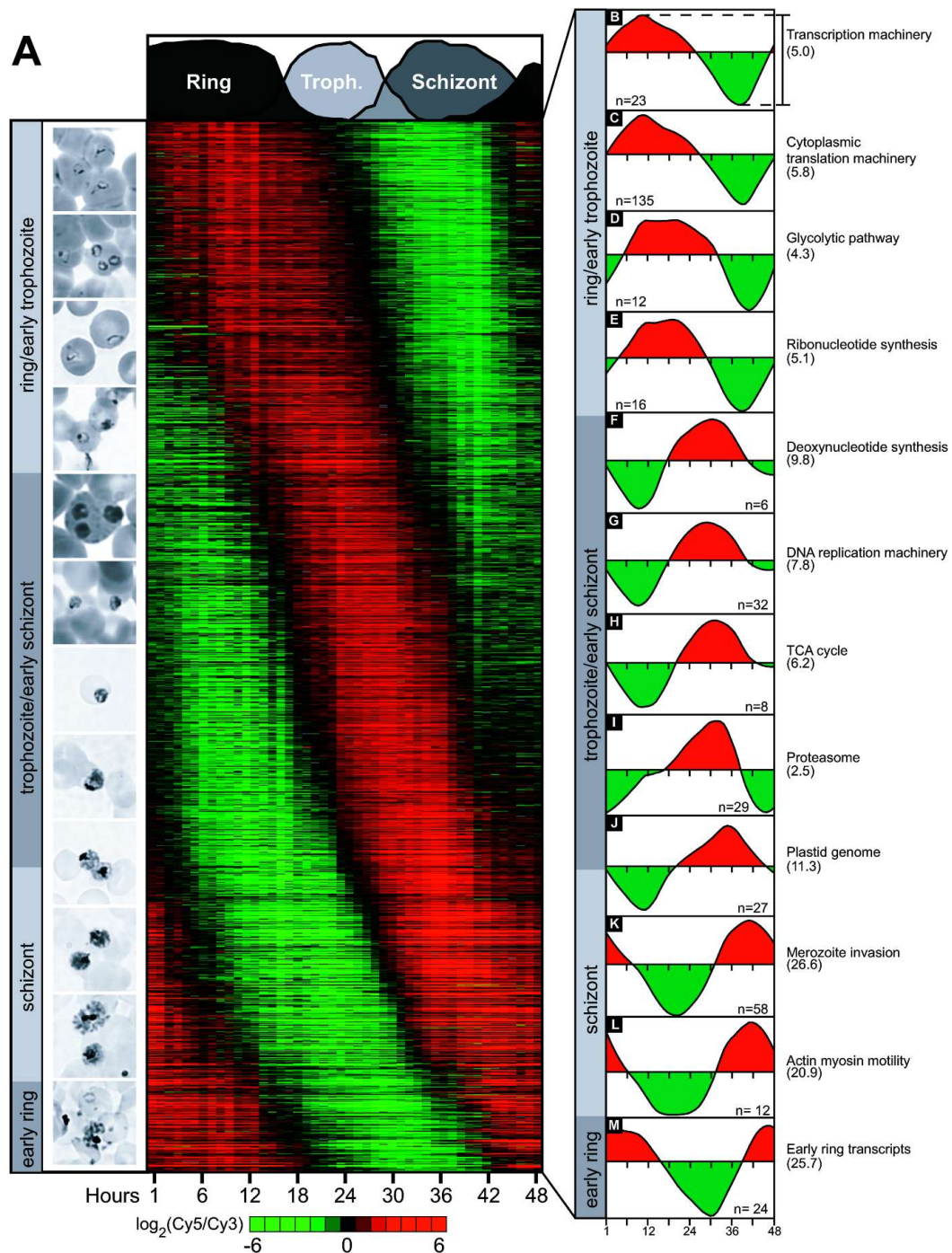


Figure 4: A) Phaseogram of the transcriptome of the asexual erythrocytic cycle, ordering the transcriptional profile of genes measured using a DNA microarray at 1-hour interval throughout the 48-hour cycle. B-M) Average expression profiles for gene expression in different biochemical processes and functions. Image taken from Bozdech *et al.*³¹

This highly regulated transcription requires a high level of control. In most organisms, transcription is controlled by transcription factors – proteins which initiate and regulate transcription. However only a few transcription factors have been identified in *P. falciparum*,³²⁻³⁵ and therefore other modes of transcriptional control must be deployed by the parasite. Epigenetic regulation is emerging as one of the most important methods of transcriptional control deployed in the parasite.

1.2.3. Post-Translational Modifications

As previously mentioned, the local structure of chromatin determines if genes are transcriptionally active. This structure is regulated by epigenetic mechanisms such as DNA methylation, non-coding RNAs (ncRNAs), or histone post-translational modifications (PTMs), which can therefore activate or block transcription of the genome.³⁶ Histone PTMs are widely distributed throughout the parasite genome, and hence are the main epigenetic processes accountable for transcription regulation in *P. falciparum*.³⁷ PTMs are covalent modifications to residues on the histone proteins, commonly on the tails of histones 3 and 4, and include methylation, acetylation, phosphorylation and ubiquitination, the most studied histone PTMs to date.³⁸ Acetylation, the addition of an acetyl group to a lysine residue, and methylation, the addition of one, two or three methyl groups to lysine or arginine residues, are both common PTMs in *P. falciparum*,²⁸ some of the known sites of which are shown in Figure 5.³⁰

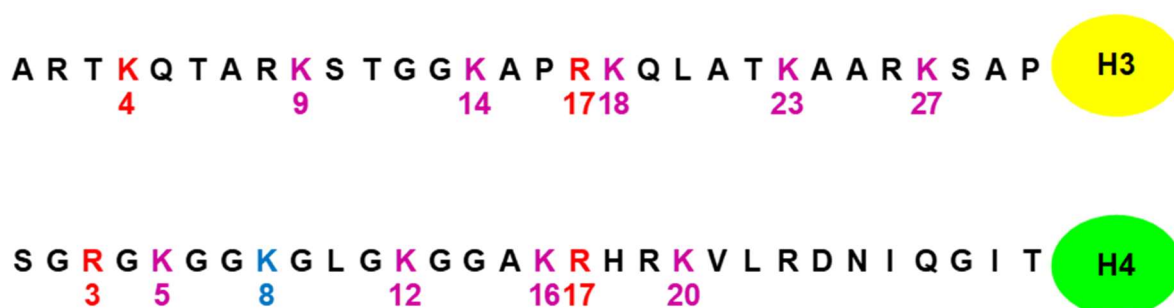


Figure 5: Sites of methylation (red), acetylation (blue) or both (purple) on the N-terminal tails of histones 3 and 4.

Since histones contain a large number of basic residues, they are positively charged at physiological pH, while the DNA is negatively charged. This is the reason why histones are able to condense with DNA. However, as a result of lysine acetylation, the overall positive charge of the histone tails decreases, reducing their affinity for negatively charged DNA generating as a result a transcriptionally active state of chromatin, euchromatin.³⁹ Histone methylation on the contrary does not affect the positive charge of the histones, and different methylation markers can be associated with either transcriptionally active or inactive chromatin.⁴⁰ For example methylation at histone 3 lysine 9 (H3K9) has been linked to gene silencing and heterochromatin,⁴¹ whilst trimethylation at histone 3 lysine 4 (H3K4me3) has been linked to transcriptional activation (although dimethylation is associated with both active and inactive genes).⁴² These histone PTM marks can also act as 'signals' for the recruitment of further proteins, which can in-turn impact gene expression.⁴³ These markers play an important role in transcriptional control in *P. falciparum*.

1.2.4. Epigenetic Markers and Transcriptional Control in *P. falciparum*

PTMs, in particular methylation and acetylation, are highly dynamic throughout the parasite's lifecycle.^{28,30} Previously, transcriptional activation markers such as H3K9ac and H3K4me3 have been found to be highly dynamic, and tightly correlated with overall transcription levels in the parasite.²⁸ Recent quantitative chromatin proteomics studies have shown that the dynamic nature of these marks is a global phenomenon throughout the life-cycle, with stage-specific markers in both the asexual erythrocytic lifecycle and in gametocyte development (Figure 6).³⁰ For example, the transcriptionally active marker H3K4me3 shows relatively low abundance in the ring-stage and trophozoite parasites, but high abundance in schizont parasites, whilst H3K9ac peaks in the trophozoite parasites. In contrast, gametocytes are associated with the high abundance of a different set of PTMs, for example H3K27me3, a marker that promotes gene silencing and heterochromatin formation. This dynamic and stage-specific nature of PTMs in *Plasmodium* suggests a vital role for these markers in

transcriptional control throughout the lifecycle. Additionally, some of these histone PTMs in *Plasmodium* localise in specific genomic regions rather than throughout the genome, including H3K9me3.^{28,37}

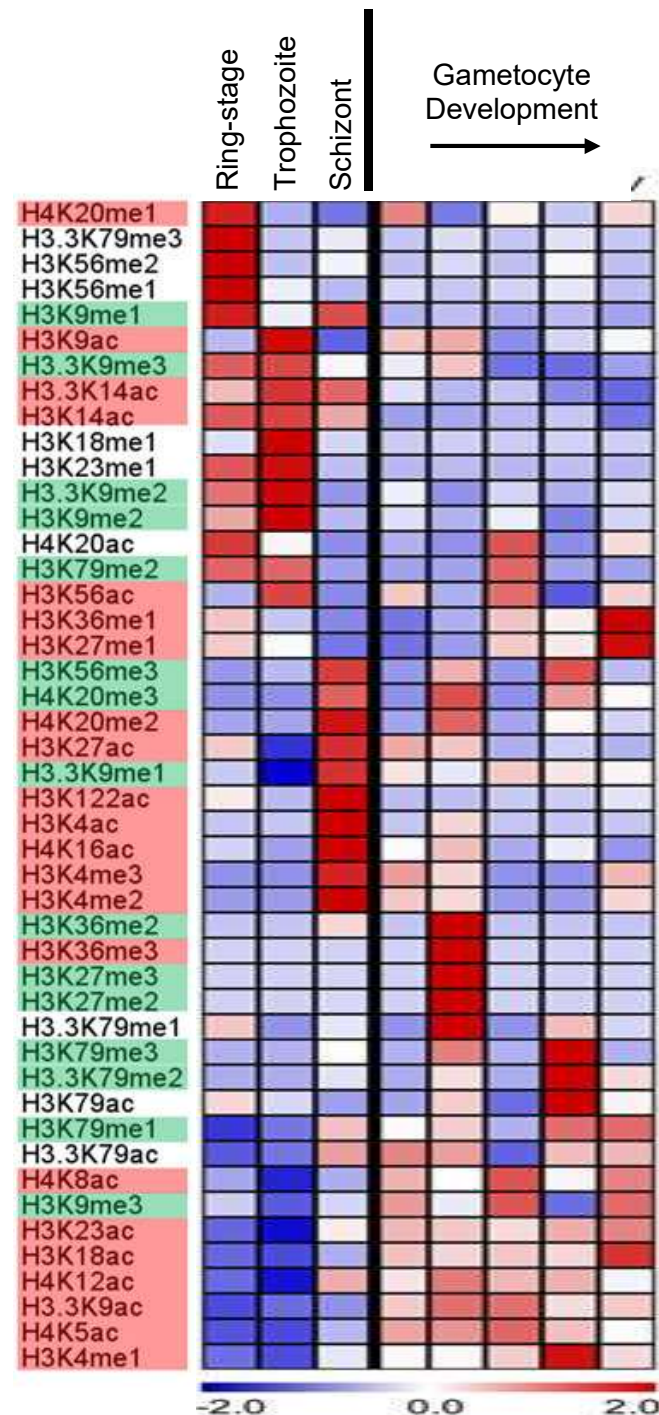


Figure 6: The relative abundance of histone methylation and acetylation marks throughout the *Plasmodium falciparum* lifecycle, during the asexual erythrocytic lifecycle and gametocyte development, from low (blue) to high (red). Marks associated with transcriptionally active euchromatin are shown in red; those associated with heterochromatin are shown in green. Image adapted from Coetzee *et al*,³⁰ under the Creative Commons licence.

1.2.4.1. Antigenic Variation

As well as overall studies of PTMs in *Plasmodium*, these markers have been found to be involved in a number of processes vital to parasite survival, including antigenic variation.

Antigenic variation is the process by which the *Plasmodium* parasite avoids detection by the human immune system. As previously mentioned, during the blood stage of the lifecycle, cytoadherence of infected red blood cells (iRBCs) to the vascular lining can occur, which allows the parasite to avoid clearance by the spleen. In *P. falciparum*, the antigens responsible for this cytoadherence are members of the *P. falciparum* Erythrocyte Membrane Protein-1 (*PfEMP1*) family. *PfEMP1* is expressed on the surface of the iRBCs, to allow them to bind to the vascular lining.⁴⁴ Being on the surface of the iRBCs, these proteins can trigger an immune response. However, the parasite has developed a mechanism to avoid detection by the host's immune system, by expressing a large number of clonally variant *PfEMP1* proteins. These different proteins are encoded for by a family of ≈ 60 *var* genes, that are activated mutually exclusively – only one *var* gene is active at a time, whilst the rest remain silent, allowing the parasite to switch between variants of *PfEMP1*, therefore evading recognition by the host's antibodies.⁴⁵

During the asexual erythrocytic cycle, *var* gene transcription peaks in early-ring stage parasites, and goes silent in the later stages.⁴⁶⁻⁴⁸ A number of epigenetic marks have been linked to both active and silent transcriptional states of *var* genes at different points in the cycle,²⁹ as shown in Figure 7. Thus, actively transcribed *var* genes have been found to possess highly enriched levels of H3K4me2, H3K4me3 and H3K9ac during the ring stage.⁴⁹ These markers are linked to euchromatin, and transcriptional activation. As the asexual cycle continues and the parasite matures to its trophozoite and schizont forms, the *var* gene transcription is silenced. Nevertheless, the same *var* gene persists through multiple erythrocytic cycles and is activated again after re-invasion, in the ring stage of the next cycle. Interestingly, Lopez-Rubio *et al.* found that although H3K4me3 and H3K9ac marks are lost on transcription silencing, high levels of H3K4me2 remain in the 'poised' *var* gene, acting as

a mark for its reactivation in the next cycle.⁴⁹ Conversely, H3K9me3 has been associated with silenced *var* genes, where high levels of this heterochromatin-associated mark can be detected.⁴⁹⁻⁵⁰ H3K9me3 has been shown to localise to the *var* genes in *Plasmodium*, and is unique from repressive marks elsewhere in the genome.³⁷

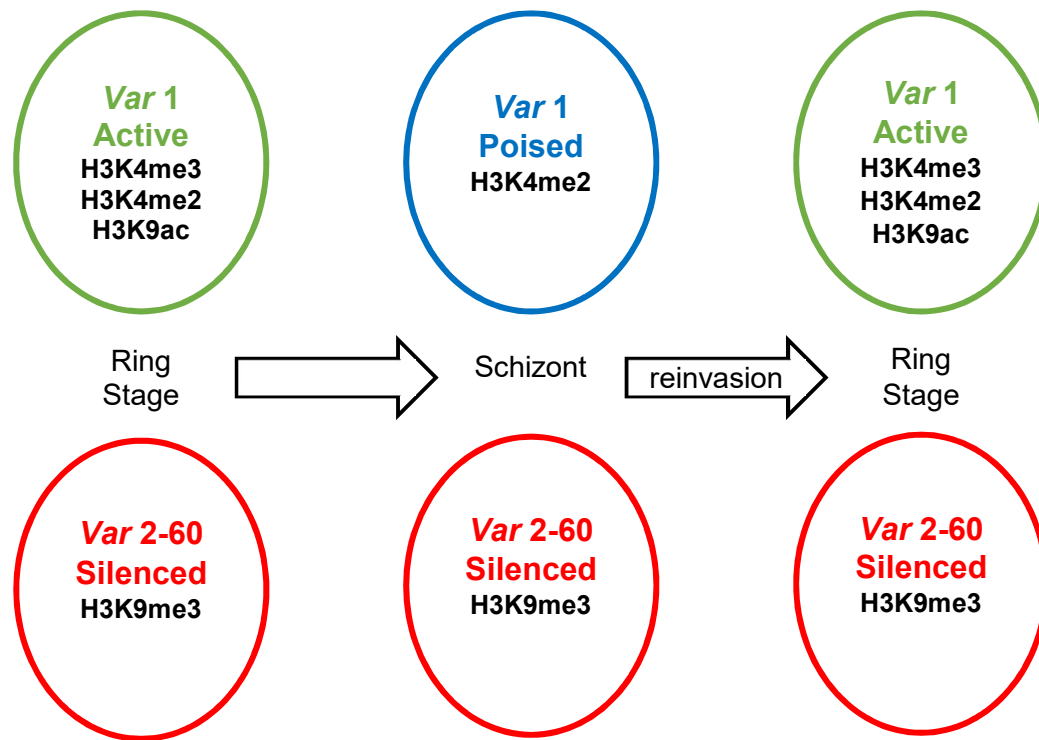


Figure 7: Epigenetic markers for *var* gene transcriptional states. Only one *var* gene is active at a time, and is persistent through multiple lifecycles.

1.2.5. Epigenetic Machinery: Readers, Writers and Erasers

As PTMs determine the structure of chromatin and therefore affect transcriptional regulation, the proteins involved in introducing, removing or detecting these modifications are key epigenetic regulators likely involved in parasite survival. These epigenetic regulators can be divided into 3 categories: 'writers', which are the proteins responsible for introducing the modifications, 'erasers' that remove them, and 'readers' that detect and bind to these modifications (Figure 8).

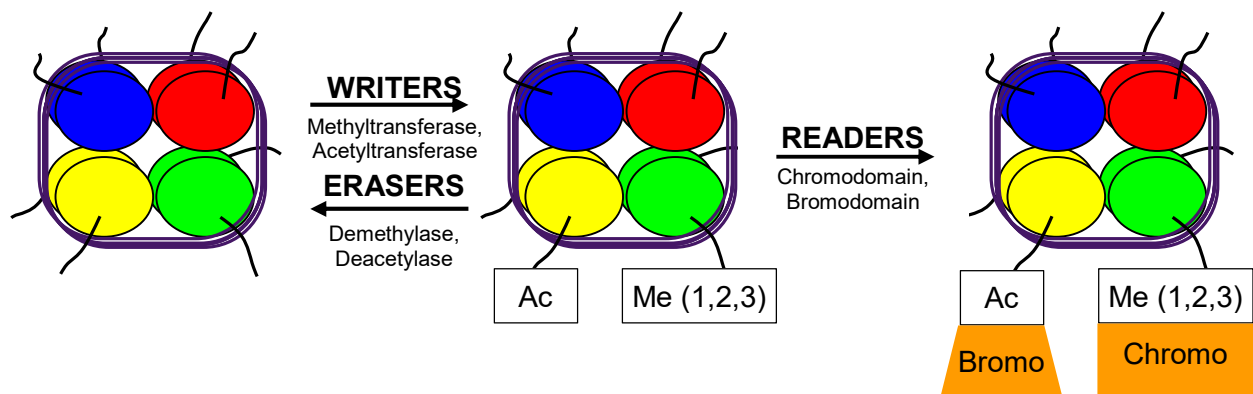


Figure 8: 'Writer' proteins such as histone lysine methyltransferases (HKMTs) or histone acetylases (HATs) introduce these modifications. 'Eraser' proteins such as histone demethylases (HMTs) or histone deacetylases (HDACs) remove these marks. 'Reader' proteins detect these marks via specific domains, such as the chromodomain or the bromodomain.

The writers, readers and erasers responsible for acetylation and methylation of *P. falciparum* histones are summarised in Table 1. These marks come from the introduction of an acetyl group or a methyl group to the lysine residues of the histone tails (and arginine residues in the case of methylation). The writers responsible for these marks are histone acetyltransferases (HATs) or histone methyltransferases (HMTs), respectively. Thus, the eraser proteins that remove these modifications from the above-mentioned residues are histone deacetylases (HDACs) and demethylases (HDMs). Finally, 'reader' proteins recognise the modified residues and bind to them via a specific domain within their structure. For example, bromodomain-containing proteins will recognise acetylated lysine residues on histones, whilst chromodomain-containing proteins will recognise methylated ones. Then, these readers can influence transcriptional control directly or by recruiting further proteins.

Table 1: Summary of Writer, Eraser and Reader Proteins for Methylation and Acetylation of Histones

	Acetylation	Methylation
Writers	Histone Acetyltransferases (HATs)	Histone Methyltransferases (HMTs) Lysine Methyltransferases (HKMTs) Arginine Methyltransferases (HRMTs)
Erasers	Histone Deacetylases (HDACs)	Histone Demethylases (HDMs)
Readers	Bromodomain	Chromodomain, Tudor domain

1.2.5.1. Acetylation

Histone acetyltransferases, or HATs are ‘writer’ proteins that catalyse the addition of an acetyl group from acetyl-CoA to lysine residues, as illustrated in Figure 9.

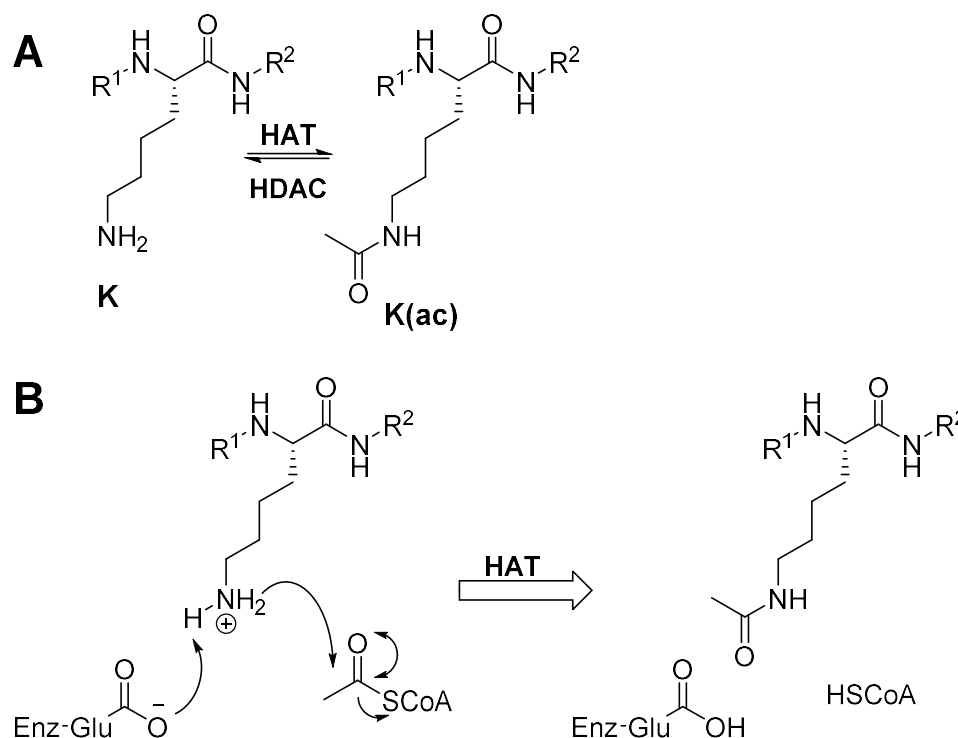


Figure 9: A) Lysine acetylation by writer proteins histone acetyl transferases (HATs) and deacetylation by eraser proteins histone deacetylases (HDACs); B) transfer of the acetyl group from acetyl-CoA to the lysine residue in the active site of HATs, with glutamic acid deprotonating the lysine.

HATs can be divided into families, based on sequence homology and function. There are several families of HAT, including GNATs (Gcn5-related N-acetyltransferases) that are characterised by the additional presence of a bromodomain and conserved catalytic motifs, and the MYST family that are characterised by the presence of a zinc finger and a chromodomain.⁵¹ The presence of ‘reader’ domains in these proteins may suggest a co-operative relationship between different epigenetic marks.

In *P. falciparum*, eight putative HATs have been identified.⁵² *PfGCN5* is a member of the GNAT family of HATs that acetylates H3K9 and H3K14,⁵³ and its expression is tightly correlated with H3K9ac and gene activation in the erythrocytic lifecycle.⁵⁴ Another is

*Pf*MYST, which acetylates H4K5, H4K8, H4K12 and H4K16, and is an essential protein in *P. falciparum*: complete knock-down attempts have been unsuccessful, whilst over-expression of an inactive form of *Pf*MYST leads to the disruption of cell cycle progression, and increases sensitivity towards DNA damage.⁵⁵

The 'eraser' proteins that remove acetyl marks are histone deacetylases, or HDACs. HDACs can be divided into a number of classes depending on sequence and function. These include class I, class II and class III. Class I and II HDACs are both Zn⁺ dependent, whilst class III HDACs are NAD⁺ dependent.

Five HDACs, belonging to classes I, II and III, have been identified in *P. falciparum*.⁵² To highlight the important role of these HDACs in the transcriptional regulation in *P. falciparum*, Chaal *et al.* showed that treatment of the parasite with a promiscuous HDAC inhibitor induced significant transcriptional changes throughout the asexual erythrocytic blood cycle, including a 90% reduction in growth, and induction of genes that are usually suppressed in different lifecycle stages.⁵⁶

HDACs are involved in a number of essential processes in *P. falciparum*. The process in which trophozoites develop into sexual gametocytes instead of carrying on with their asexual maturation to form schizonts is essential for the progression of the parasite lifecycle (Figure 1). Only a small number of parasites undergo gametocyte conversion in each cycle, but these gametocytes are needed for the transmission of the parasite back to the mosquito host. *Pf*AP2-G has been found to be the major transcription factor involved in the commitment of the parasite to sexual differentiation,⁵⁷⁻⁵⁸ and this transcription factor has been found to be regulated by the class II HDAC *Pf*Hda2: knock-down of *Pf*Hda2 dysregulated around 70% of gametocytogenesis-related genes, including the one encoding for *Pf*AP2-G itself, and increased levels of gametocyte conversion.⁵⁹

As previously mentioned, antigenic variation, the process by which the parasite avoids detection by the human immune system, is vital for parasite survival. *Pf*SIR2A and *Pf*SIR2B

are class III HDACs involved in antigenic variation and *var* gene silencing, and the knock-out of either enzyme results in the simultaneous expression of multiple *var* genes.⁶⁰ *PfSIR2A* is also involved in the regulation of the transcription of ribosomal RNA, the part of the ribosome responsible for protein synthesis.⁶¹

The 'reader' proteins that recognise acetylated lysine marks contain bromodomains, a number of which have been identified in *P. falciparum*.⁶² Of these, two have been characterised: HDAC *PfGCN5*,⁵³ and *PfBDP1*, a bromodomain-containing protein that binds to chromatin at genes related to invasion.⁶³ During the erythrocytic lifecycle the *Plasmodium* parasite must invade RBCs and go through multiple cycles of re-invasion (Figure 1B). *PfBDP1* is vital for this process: conditional knock-down of *PfBDP1* leads to down-regulation of critical invasion-related genes, resulting in serious disruption to invasion.⁶³

1.2.5.2. Methylation

Methylation is another important PTM in *P. falciparum*. Histone lysine methyltransferases, or HKMTs are one of the previously mentioned classes of 'writer' protein that catalyse the addition of one, two or three methyl groups to lysine residues of histones. These different methylation states are shown in Figure 10a.

With the exception of the DOT1 class,⁶⁴ all HKMTs contain a conserved catalytic region known as the SET (from the Su(var)3-9, Enhancer-of-zeste and Trithorax proteins of *Drosophila*) domain. The domain is over 100 amino acids long, and will target histone or non-histone substrates, depending on the protein.⁶⁵⁻⁶⁶ The SET domain contains the active catalytic site, which binds the lysine residue such that the nucleophilic attack can take place, as well as the cofactor SAM (S-adenosyl-L-methionine), which provides the methyl group. The nucleophilic lysine residue attacks the methyl group on the positively charged SAM cofactor, releasing the methylated lysine and S-adenosyl-L-homocysteine (SAH). This is shown in Figure 10b.

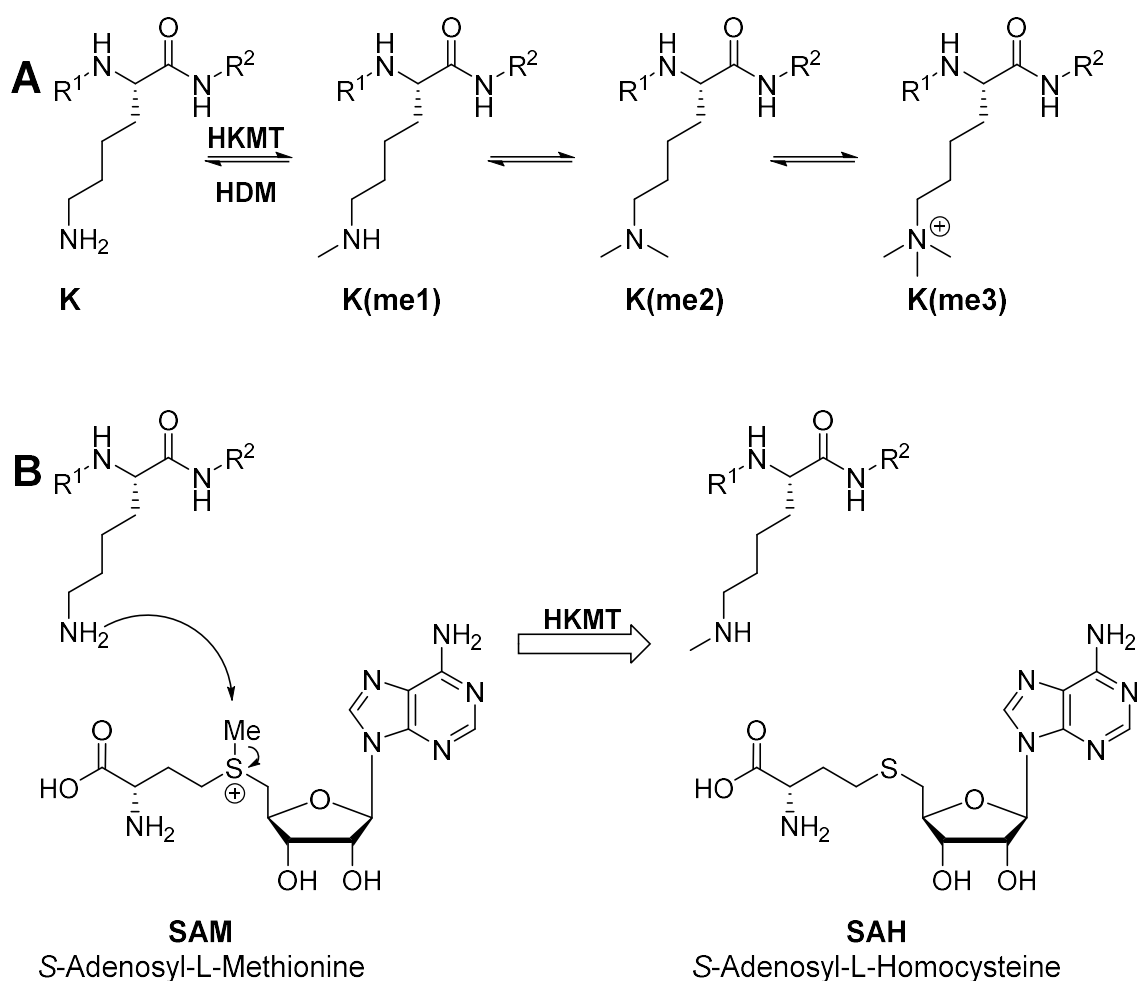


Figure 10: Lysine methylation by writer proteins histone lysine methyl transferases (HKMTs) and demethylation by eraser proteins histone demethylases (HDMs): A) the different methylation states of lysine; B) transfer of the methyl from SAM to the lysine by nucleophilic attack in the active site of HKMTs.

There are ten HKMTs in *Plasmodium falciparum* predicted from the genetic sequence, listed in Table 2. All of these are SET domain-containing proteins, and six of them are thought to be essential during the blood stage.⁶⁷⁻⁶⁹ To date, only *PfSET7* has been successfully expressed as recombinant protein, and has been shown experimentally to methylate H3K4 and K3K9, with preference for substrates with H3K14ac markers.⁷⁰

Table 2: The 10 predicted SET-domain containing HKMTs in *Plasmodium falciparum*⁶⁷⁻⁷⁰

	Length (amino acids)	Predicted Specificity	Essential
<i>PfSET1</i>	6753	H3K4me1-3	Yes
<i>PfSETvs</i> (<i>PfSET2</i>)	2548	H3K36me2-3	No
<i>PfSET3</i>	2399	H3K9me2-3	Yes
<i>PfSET4</i>	1114	H3K4	No
<i>PfSET5</i>	178	Unknown	No
<i>PfSET6</i>	509	H3K4	Yes
<i>PfSET7</i>	810	H3K4, H3K9*	Yes
<i>PfSET8</i>	1186	H4K20me1-3	No
<i>PfSET9</i>	1674	Unknown	Yes
<i>PfSET10</i>	2329	H3K4	Yes

*Experimentally determined⁷⁰

The roles of some of these HKMTs have been elucidated. *PfSETvs*, also known as *PfSET2*, methylates H3K36. Jiang *et al.* found that knocking out this SET2 gene resulted in simultaneous transcription and expression of virtually all *var* genes and consequently surface proteins.⁶⁹ Hence inhibiting this enzyme may be a viable route for therapeutics, removing the ability of the parasite to hide from the human host. *PfSET10* has also been found to be involved in *var* gene regulation: it co-localises with active *var* genes and is also required for maintaining these genes in a ‘poised’ state for reactivation in the next lifecycle.⁶⁸ However, overall relatively little is known about the *Plasmodium* HKMTs, with the biological roles of many remaining unknown.

Methyl marks are removed by histone demethylases (HDMs) which can be divided into two classes: JmjC domain-containing demethylases (JHDMs) and lysine-specific demethylases 1 (LSD1).⁷¹ Two JHDMs and one LSD1 protein have been identified in *P. falciparum*,^{67,72} although these have not yet been fully characterised and their roles in the parasite lifecycle remain largely unknown.

There are also a number of 'reader' proteins that recognise methyl marks in *P. falciparum*, including chromodomains and tudor domains.⁷²⁻⁷³ One of the best characterised to date is the chromodomain-containing reader protein *P. falciparum* heterochromatin protein 1 (*PfHP1*).⁷⁴ HP1 proteins are associated with heterochromatin formation and transcriptional control, binding selectively to H3K9me2 and H3K9me3.⁷⁵ *PfHP1* has been linked to the mutually exclusive expression of *var* genes.⁷⁴ As an H3K9me3 reader, *PfHP1* not only protects these marks from the action of demethylases, which would remove the methyl groups and hence activate transcription, but also recruits further HMTs promoting heterochromatin formation.⁷⁶ In fact, Brancucci *et al.* found that knock-down of *PfHP1* led to a reduction in the local levels of H3K9me3 and to the simultaneous activation of multiple *var* genes.⁷⁷ *PfHP1* is also involved in gametocyte conversion, and the regulation of transcription factor *PfAP2-G*: the depletion of *PfHP1* triggers significantly higher levels of gametocyte conversion.⁷⁷ As previously mentioned *PfHP1* binds to the silencing mark H3K9me3 favouring heterochromatin formation and maintenance, and therefore it may be vital to keeping gametocyte conversion genes silent.

1.2.6. Epigenetics and Drug Discovery

1.2.6.1. Epigenetic Therapeutics in Cancer

Epigenetic modifications regulate cellular pathways, and so it is unsurprising that they are often dysregulated in diseases. This has been found to be the case in cancer, as well as other diseases including asthma, diabetes and neurological disorders.⁷⁸ This has led to increasing research into these mechanisms as targets for novel therapeutic intervention. In cancer in particular, there is emerging evidence of an intrinsic link between the roles of genetics and epigenetics in the disease.⁷⁹⁻⁸⁰ Many epigenetic alterations have been observed in cancer, including altered levels of histone and DNA methylation, and histone acetylation.⁸¹

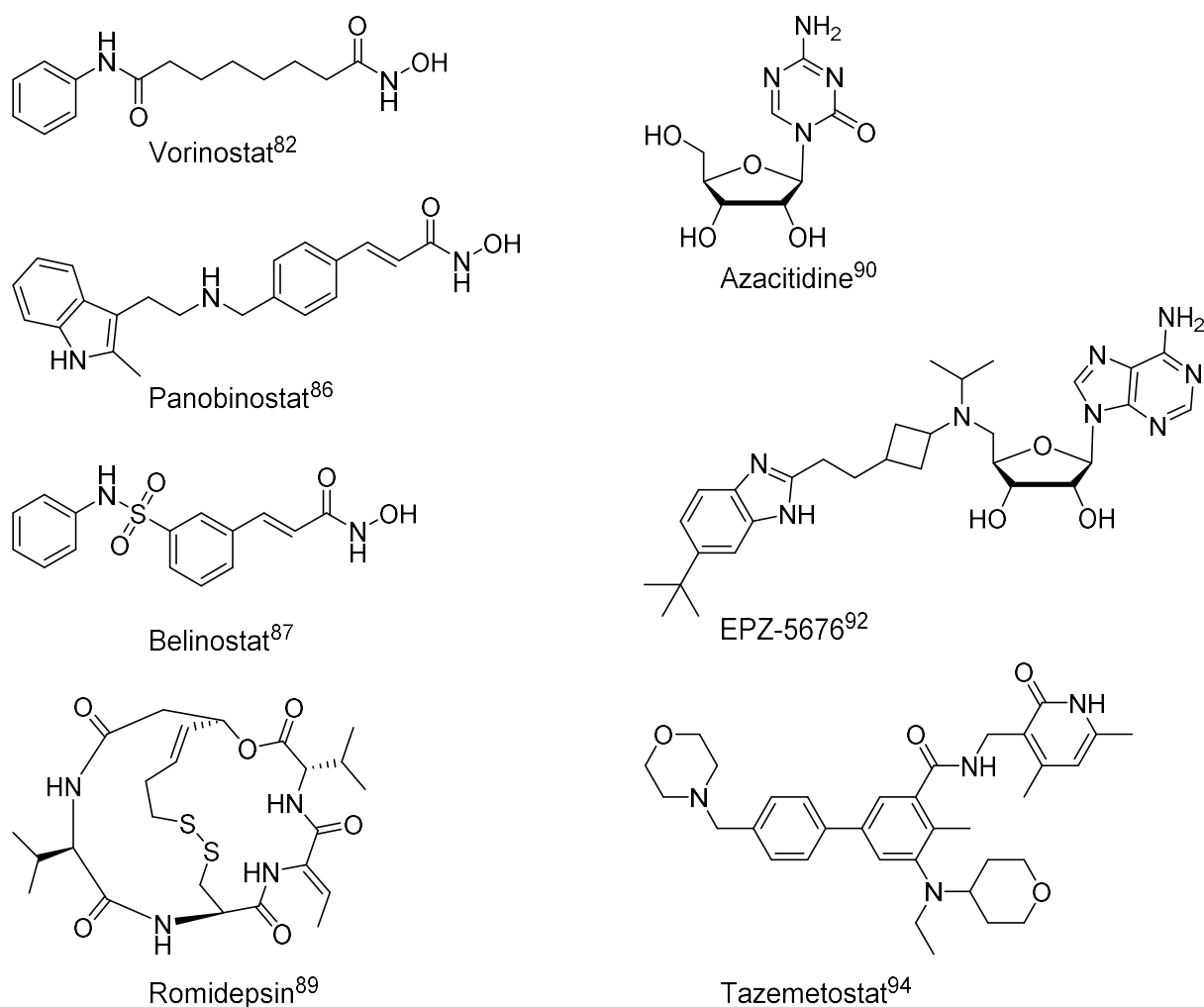


Figure 11: Chemical structures of epigenetic inhibitors that are FDA approved or in clinical trials for cancer therapeutics.

One successful example of a therapeutic targeting an epigenetic mechanism in cancer is Vorinostat (suberoylanilide hydroxamic acid, SAHA) (Figure 11).⁸² This hydroxamic acid is an HDAC inhibitor that was approved by the US Food and Drug Administration (FDA) in 2006, for the treatment of cutaneous T-cell lymphomas. As mentioned before, HDACs remove acetyl groups from the lysine side chains in histone tails or other targets. Histone deacetylation is linked to gene silencing through the increase in the strength of the DNA-histone interaction due to the increase in charge on the deacetylated histone, or due to the recruitment of specific effectors.⁸³ The mechanisms by which HDAC inhibitors exert anti-tumour activity are complex. HDACs have many substrates, including many non-histones,⁸⁴

and hence their inhibition produces a large number of down-stream effects, including the up-regulation of tumour suppressors and the promotion of apoptosis.⁸⁵

Vorinostat and other FDA approved drugs such as panobinostat⁸⁶ and belinostat⁸⁷ (Figure 11) bind to zinc-dependant HDACs, using the hydroxamic acid subunit for chelating the Zn atom.⁸⁸ Similarly, Romidepsin (Figure 11) acts as a pro-drug containing a di-sulfide bond that when reduced *in vivo* can bind to the zinc atom in the HDAC binding pocket.⁸⁹

Other epigenetic mechanisms have also been targeted by cancer therapeutics. For example, Azacitidine (Figure 11) is an FDA-approved drug that inhibits DNA methyltransferases.⁹⁰⁻⁹¹ A number of HKMT inhibitors are currently in clinical trials. EPZ-5676 (Figure 11) is a potent inhibitor of DOT1L, a non-SET domain containing HKMT, which has shown potent activity against mixed lineage leukemia.⁹² Tazemetostat (Figure 11) is an inhibitor of EZH2, a SET domain HKMT that methylates H3K27, which has also shown promising anti-tumour activity in clinical trials.⁹²⁻⁹⁴ The successful use of epigenetic modulators in cancer therapeutics, encourages further research efforts on these targets for other diseases, including malaria.

1.2.6.2. Targeting Epigenetics in Malaria

Given the vital role epigenetic processes play in the *Plasmodium* lifecycle, the main epigenetic modifiers - namely writers, erasers and readers - provide exciting new targets for the discovery of antimalarials with new modes of action. The most common approach has been to target HDACs, probably due to the validation of these targets in other important diseases such as cancer, where a number of inhibitors have been approved for use in the clinic (Figure 11), and due to the well-established HDAC pharmacophore.

Considering the vital role of HDACs in *Plasmodium*, the potential of these enzymes as targets for antimalarial drug discovery is clear. Taking advantage of all the research carried out for human HDACs, one common approach to targeting HDACs in malaria has been to repurpose known HDAC inhibitors that have been validated in other diseases.⁹⁵

A number of aforementioned clinically approved HDAC inhibitors, Vorinostat, Belinostat, Panobinostat and Romidepsin (Figure 11), have been tested for antimalarial activity, and have been found to possess potent nanomolar potency against asexual blood-stage parasites.⁹⁶⁻⁹⁷ Oral administration of Panobinostat to infected mice led to a reduction in parasitemia and increased survival.⁸⁶ Similarly, when the FDA-approved HDAC inhibitor Pracinostat was tested for antimalarial activity, it displayed nanomolar potency against a number of *Plasmodium* strains, as well as *in vivo* activity upon oral administration in a mouse model.⁹⁸ In all cases, hyperacetylation of histones was observed on treatment with these compounds, suggesting on-target activity.^{86,96,98}

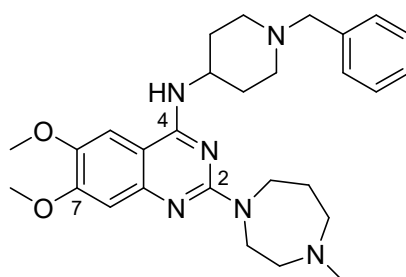
A number of research groups have tried to develop new libraries of compounds aiming at the improvement of antimalarial properties and at the achievement of selectivity for parasite over human HDACs. Thus, Hansen *et al.* developed a small library of Vorinostat derivatives containing an alkoxyamide linker, finding a few hits with nanomolar potency against multiple lifecycle stages, and with some of them displaying parasite selectivity.⁹⁹ In another approach, Patel *et al.* found 17 hydroxamate compounds out of a screen of a large library of around 2000 HDAC inhibitors, that displayed antimalarial activity alongside minimal changes in acetylation in mammalian cells.⁹⁷

HATs are also emerging as a potential novel therapeutic target in *P. falciparum*. Known promiscuous HAT inhibitors curcumin and anacardic acid inhibit recombinant *Pf*GCN5, reduce H3K9ac levels in the asexual erythrocytic cycle, and inhibit parasite growth and development,¹⁰⁰⁻¹⁰¹ and novel inhibitors of *Pf*GCN5 are now being developed.¹⁰²

HDAC inhibition has been shown to be a potential novel approach for malaria therapeutics, with other epigenetic targets also emerging as potential novel targets. The importance of epigenetics in the lifecycle means that other epigenetic processes, such as HKMT inhibition, may offer further opportunities for future drug discovery.

1.3. The BIX-01294 Compound Series

Following the success of repurposing human HDAC inhibitors for antimalarial activity, a similar approach has been attempted with *Plasmodium* HKMTs, as recombinant proteins are not available for target-based drug discovery. BIX-01294 (Figure 12) is a human HKMT inhibitor with potent antimalarial properties and some selectivity over human cell lines.¹⁰³⁻¹⁰⁴



BIX-01294
IC₅₀ (*Pf*3D7) = 43 nM
MW = 490.65
cLogP = 3.82
tPSA = 64.9

Figure 12: Chemical structure of human HKMT inhibitor BIX-01294 with potent antimalarial properties: IC₅₀ = 43 nM against *Pf*3D7 measured by a 3-day SYBR Green I assay.

BIX-01294 was discovered when a high-throughput screen of 125,000 compounds was used to find inhibitors of human HKMT G9a, which dimethylates H3K9¹⁰⁵ and is up-regulated in a number of cancers including leukaemia, liver, prostate and ovarian cancer.¹⁰⁶ It was found to inhibit H3K9me2 methylation *in vitro*, and to reduce H3K9me2 levels in cellular assays.¹⁰⁷ It is non-competitive with SAM, binding to the substrate pocket where it forms a number of hydrophobic and hydrogen bonding interactions.¹⁰⁷⁻¹⁰⁸ Since its discovery, various attempts have been made to improve the potency and selectivity of the compound. The introduction of a lysine mimic in position 7 has been found to increase potency, by occupying the lysine pocket in the active site.¹⁰⁸ BIX-01294 and diaminoquinazoline analogues have also been found to inhibit a range of other human HKMT enzymes – GLP,¹⁰⁹ SETD8¹¹⁰ and EZH2¹¹¹, which suggests there is potential to ‘repurpose’ these compounds for other HKMTs.

1.3.1. Antimalarial activity of BIX-01294

In order to pursue HKMTs as a novel antimalarial target, BIX-01294 and a synthesised focussed library were assessed for antimalarial properties. The inhibitors exhibited nanomolar cytotoxicity against both sensitive and drug resistant strains, and clinical isolates of *P. falciparum* and *P. vivax*.¹⁰⁴ Additionally the series shows some specificity over human cell lines, and is active against all stages of the 48-hour asexual erythrocytic lifecycle of *P. falciparum*,¹⁰⁴ as well as early and late-stage gametocytes.¹¹²

The series also shows an unprecedented ability to ‘awaken’ hypnozoites in *P. vivax*, which can remain dormant in the liver and cause relapse malaria.¹¹³ There is currently only one treatment for this dormant malaria, primaquine,¹¹⁴ however this can cause haemolysis in people with glucose-6-phosphate dehydrogenase deficiency, which is prevalent in Sub-Saharan Africa.¹¹⁵ Whilst exposure to this series at higher concentrations (100 – 300 nM) had no effect, exposure at low concentrations (10 nM) accelerated hypnozoite activation in *P. cynomolgi* (a primate strain) cultures, as shown in Figure 13,¹¹³ which presents an opportunity to further study this life-cycle stage, and could also be used as part of an ‘awaken and kill’ therapeutic strategy.

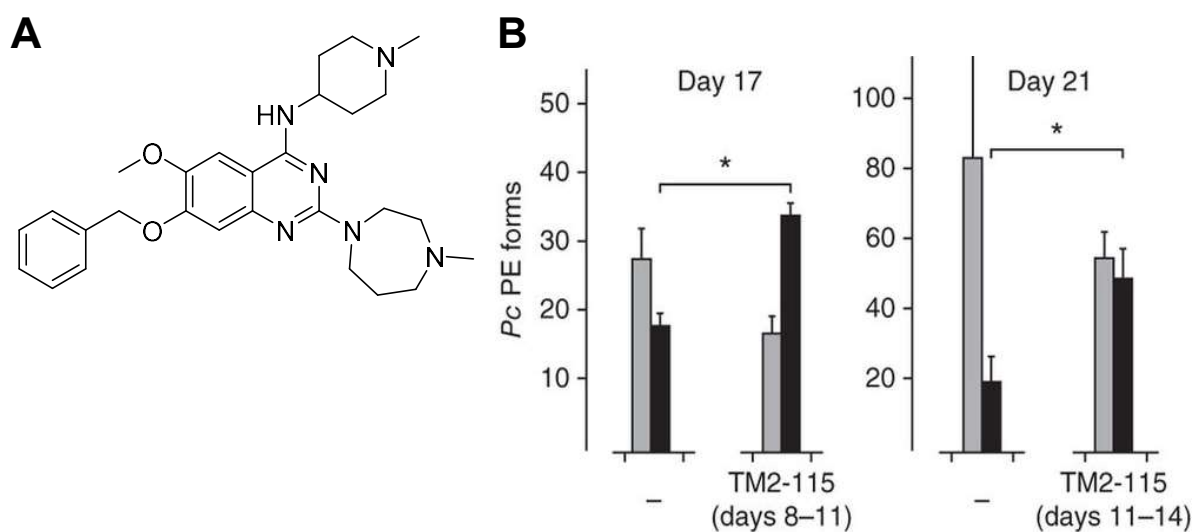


Figure 13: A) The structure of TM2-115, the diaminoquinazoline used in the hypnozoite experiments. B) Figure from Dembélé *et. al.*¹¹³ *P. cynomolgi*-infected cultures (under Matrigel) treated from days 5 - 8 or days 11 - 14 after sporozoite inoculation with 10 nM. Hyp = hypnozoites, Sch = schizonts.

As well as acting on a number of lifecycle stages, these compounds have a number of exciting *in vivo* effects. The compounds are orally available,¹¹² and active in mice against both *P. berghei* (a rodent strain), and a humanised mouse model of *P. falciparum*.^{104,112} Importantly the series does not inhibit the human cardiac voltage-gated potassium channel Ether-a-go-go Related Gene (hERG) at relevant concentrations,¹¹² which has previously been a concern for quinazolines.¹¹⁶

Interestingly, other similar quinazoline structures have also been found to have antimalarial activities (Figure 14),¹¹⁶⁻¹²⁰ although differences in the phenotypic effects of these compounds suggests differences in the mode of action. The scaffold may be promiscuous, with potential for similar off-targets between these compounds.

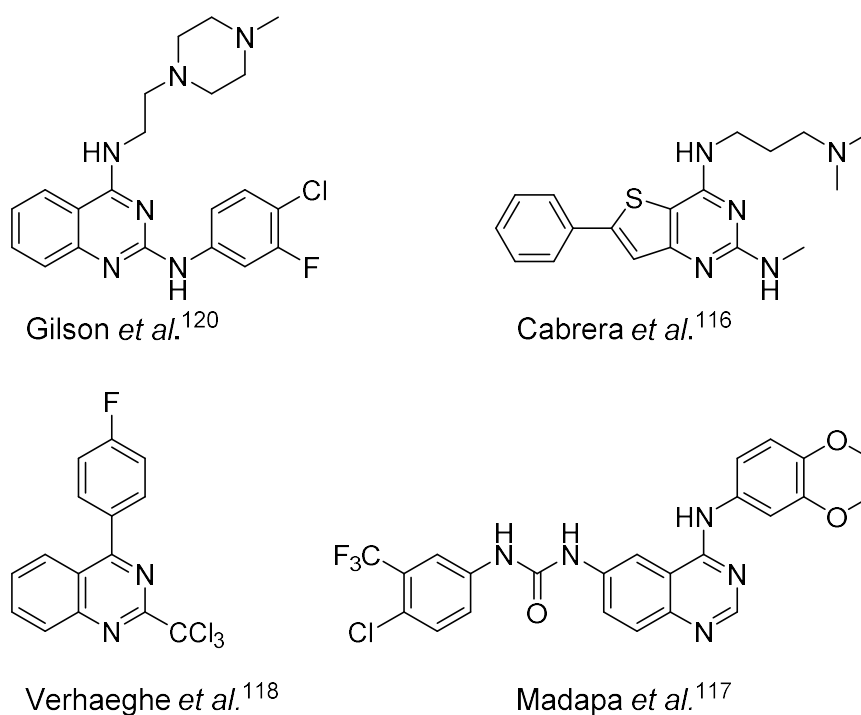


Figure 14: Chemical structures of other quinazoline scaffolds with antimalarial activity.

Overall the compound series around BIX-01294 display exciting phenotypic effects, displaying rapid antimalarial activity against all blood stages and exciting *in vivo* properties. This offers a potential avenue for future antimalarial therapeutics, although the mechanism

of action has not been confirmed. The compounds are known human HKMT inhibitors, and HKMTs are known to be vital throughout the parasite lifecycle, so it is possible that HKMT inhibition is accountable for the phenotypic effects of this compound series in *Plasmodium*. However, the full complement of *Plasmodium* HKMTs are not available: only PfSET7, which has been successfully expressed and purified recently,⁷⁰ and has been shown not to be the target of this compound series (unpublished results). Hence the direct activity of these compounds against the other PfHKMTs cannot yet be measured. Indirect approaches have shown a dose-dependent decrease in the methylation levels,¹⁰³⁻¹⁰⁴ (Figure 15), but further evidence is needed to validate these inhibitors are on-target and to confirm if they are responsible for the observed phenotypic effects.

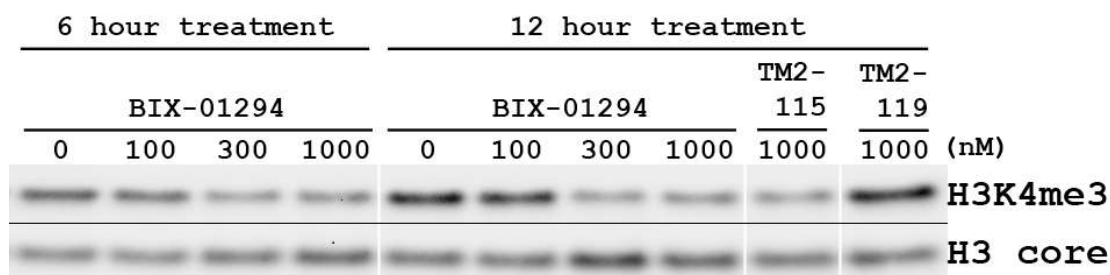


Figure 15: Image taken from Malmquist *et al.*¹⁰⁴ Western blots showing H3K4me3 levels compared to total histone H3 levels in asynchronous *P. falciparum* treated with different concentrations of BIX-01294, TM2-115 or TM2-119 (an inactive derivative). Reduction of methylation is observed on treatment with BIX-01294 and TM2-115, with dose-dependent reduction on treatment with BIX-01294.

1.4. Proteomics

There are two major approaches to drug discovery: target-based screening, whereby a compound library is screened against a specific target, or phenotypic screening, where it is screened against a whole cell or organism.¹²¹ The latter approach does not rely on the availability of recombinant protein, and considers the complexity of the target from the start, but target identification and validation can be challenging. Target identification is important for the development of new drugs, aiding medicinal chemistry and drug design.¹²² The mode of action may also help with understanding of the disease or condition being targeted,

leading to more effective therapeutics, by identifying new targets for drug discovery. Additionally, many small molecules have off-target effects that need to be identified: they may have multiple targets, which together contribute to the therapeutic effect, or they may cause unwanted side-effects.¹²³

Chemical proteomics has emerged as a versatile tool for profiling the targets of a given inhibitor.¹²⁴⁻¹²⁷ Techniques including mass spectrometry and gel-based experiments are used to identify targets of compounds within complex proteomes.

1.4.1. Activity-based protein profiling

Activity-based protein profiling (ABPP) uses functionalised chemical probes to target the active site of enzymes. The probe is based on the inhibitor of interest, and contains two functionalities: a reactive group that will form a covalent bond with the residues in the active site linking the probe to the enzyme, and a reporter group which will allow the probe-enzyme complex to be analysed (Figure 16).¹²⁸

The probe is incubated with a complex proteome (whole cells or lysates) where it binds and covalently reacts with its targets and can then be detected and analysed due to the presence of the reporter tag. In 1-step ABPP, the probe contains both the reactive functionality to bind to the target proteins, and the reporter tag for their detection, whereas in 2-step ABPP the probe contains the reactive group and a functionalisable group instead, which can be reacted with a capture reagent once the probe is bound to its targets (Figure 16).

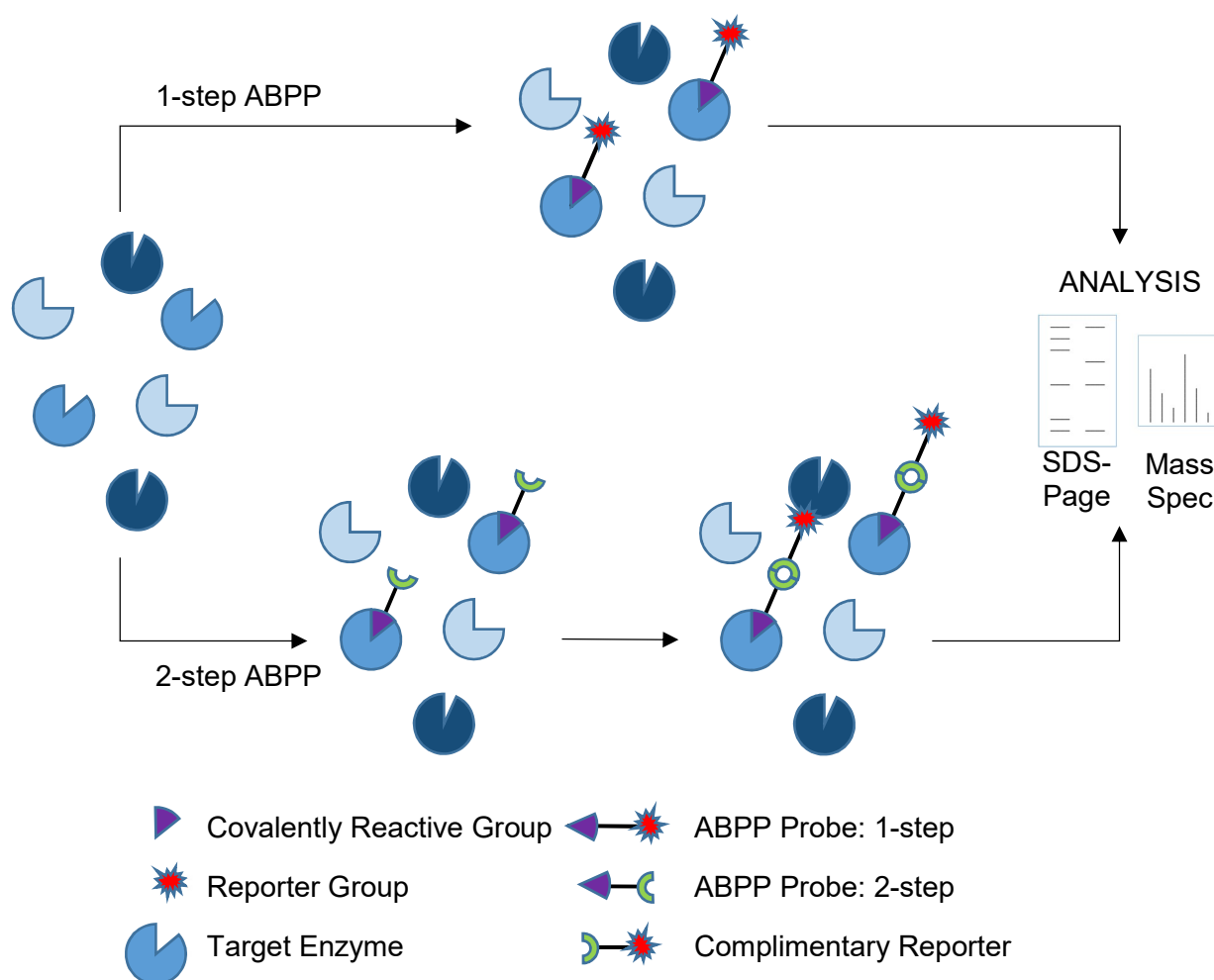


Figure 16: Schematic showing activity-based protein profiling (ABPP) via a 1 or 2 step approach. A complex proteome is incubated with a probe that will bind covalently to the target. In the 1-step approach the probe already contains a tag that allows for visualisation or identification. In the 2-step approach the reporter tag is added in a second step.

Often, these functionalisable groups on the probe and the capture reagent are an alkyne and an azide, which can be reacted using click chemistry, a robust bio-orthogonal reaction catalysed by copper (I) (Figure 17A).

The reporter tag, or capture reagent, is commonly a fluorophore or a biotin subunit (Figure 17B). A fluorophore such as TAMRA allows for direct visualisation of the target proteins after their separation using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) techniques. Biotin allows for pull-down experiments after protein enrichment by means of its strong interaction with avidin ($K_d = 10^{-15}$ M).¹²⁹ Using a solid-supported source of

avidin, such as NeutrAvidin-agarose beads, the probe-protein complexes can be enriched due to the presence of the biotin reporter tag, pulled down, and analysed by mass spectrometry.¹³⁰ In addition, a bifunctional capture reagent combining the fluorophore and the biotin, allows both analysis from one set of experiments.

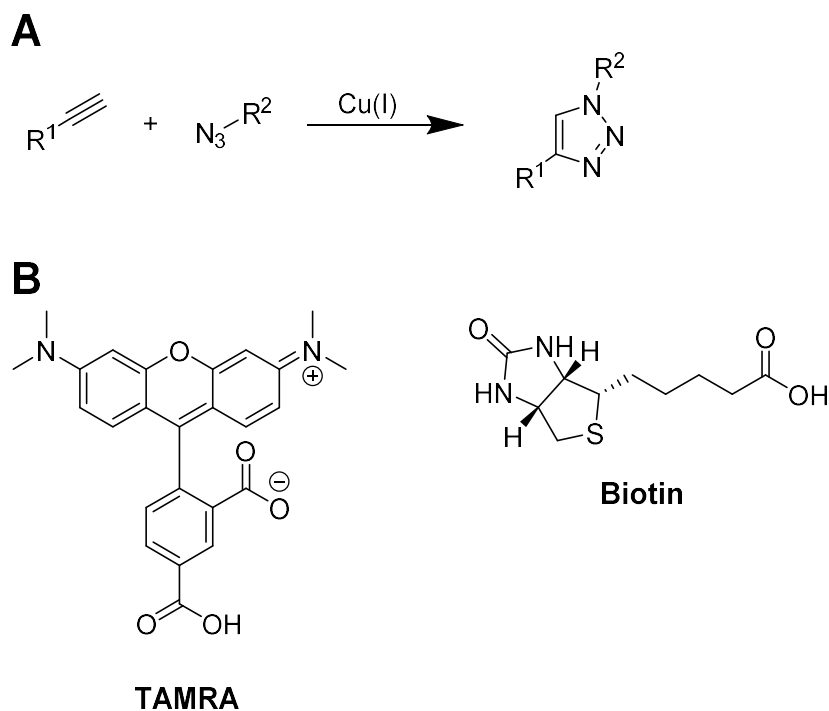


Figure 17: A) The click-chemistry reaction between an alkyne and azide catalysed by copper (I). B) The structures of TAMRA, a fluorophore, and biotin, which can be functionalised at the carboxylic acid for use as reporter tags.

Both biotin and TAMRA are bulky groups that may interfere with the affinity of the probe for its target enzymes, or have other interactions in the proteome, and cause cell permeability issues in whole-cell experiments.¹³¹ Hence the advantage of 2-step ABPP is that the probe can be made as similar as possible to the target compound, and the capture reagent added later, after the probe is already covalently bound in the enzyme's binding site. Where a whole-cell approach is used, the cells can be lysed before the capture reagent is added, so that the functionalisable group is accessible for the click chemistry reaction with the capture reagent.

One example of ABPP being used in target identification was for Orlistat by Yang *et al.* Orlistat is an FDA-approved anti-obesity drug which works as an inhibitor of pancreatic and gastric lipases.¹³² However interesting off-target effects including anti-cancer properties emerged,¹³³⁻¹³⁵ suggesting the drug had numerous targets. By making conservative modifications to Orlistat's structure adding a small alkyne handle (Figure 18), they were able to identify eight new targets involved in cancer cell biology, including GAPDH and β -Tubulin which are regulatory proteins; Annexin A2 which is involved in cell proliferation; Hsp90AB1 which is involved in protein degradation; and ribosomal proteins.¹³⁶

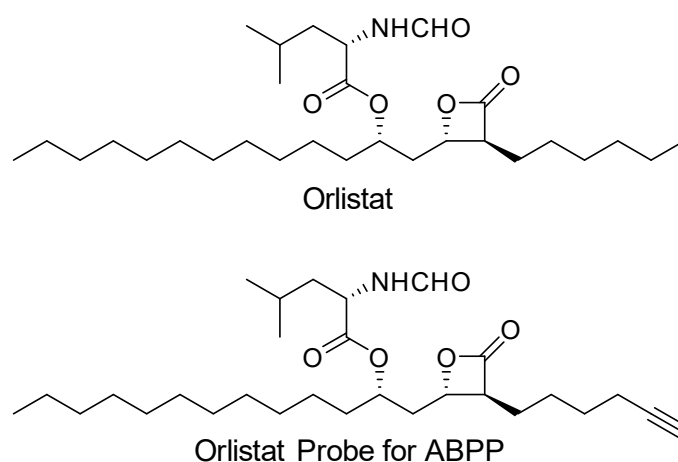


Figure 18: Chemical structures of Orlistat and the alkyne-containing probe used in ABPP to identify novel targets. Designed by Yang *et al.*¹³⁶

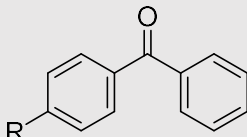
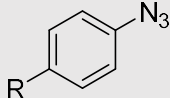
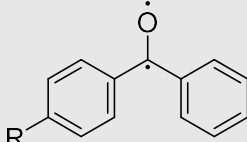
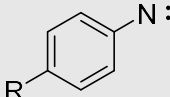
1.4.2. Photo-crosslinking probes

The ABPP described above relies on the probe binding covalently to the target protein, either by the natural reactivity of the inhibitor or by introducing highly reactive groups. However for highly reactive groups to be suitable, a high specificity and affinity for the target is required from the inhibitor to avoid reaction with other proteins before the probe-target complex has been formed.¹²⁴ An alternative is to use a probe containing a photo-crosslinking group: a functional group that upon UV irradiation will form a highly reactive species that will then bind covalently to the protein. The main difference between both approaches is that the use of a photoreactive subunit allows the use of an inhibitor that does not covalently bind to

its targets and can avoid the need to introduce highly-reactive, potentially promiscuous groups, instead allowing the covalent bond between the probe and protein to form only when UV light is applied.

Common photo-crosslinking groups include diazirines, aryl-azides and benzophenones, as illustrated in Table 3. For a photo-crosslinking group to be efficient it is important that it has a bio-compatible wavelength for activation, but it must also be bio-orthogonal, so it doesn't react before activation, or perturb the probe-protein interaction. Often, diazirines and benzophenones are preferred as the lower wavelength required for aryl-azide activation can be damaging to proteins.¹³⁷ The compact nature and highly reactive carbene intermediate of diazirines makes this is a popular choice.¹³⁸⁻¹³⁹

Table 3: Different photo-crosslinking groups that can be incorporated into photo-activatable probes. They form reactive species upon UV irradiation.¹⁴⁰

	Alkyl- or Aryl-Diazirine	Benzophenone	Aryl-azide
Photo-crosslinking group			
UV activation wavelength (nm)	350-365	350-365	254-400
Reactive group	 Carbene	 Diradical	 Nitrene
Reactivity	C-H and X-H insertion	C-H insertion	C-H and X-H insertion or ketenimine formation

Hence for an inhibitor with unknown targets, the introduction of a photo-crosslinking group and a small bio-orthogonal handle provides a powerful tool for target identification.¹⁴¹ A schematic of this methodology is shown in Figure 19.

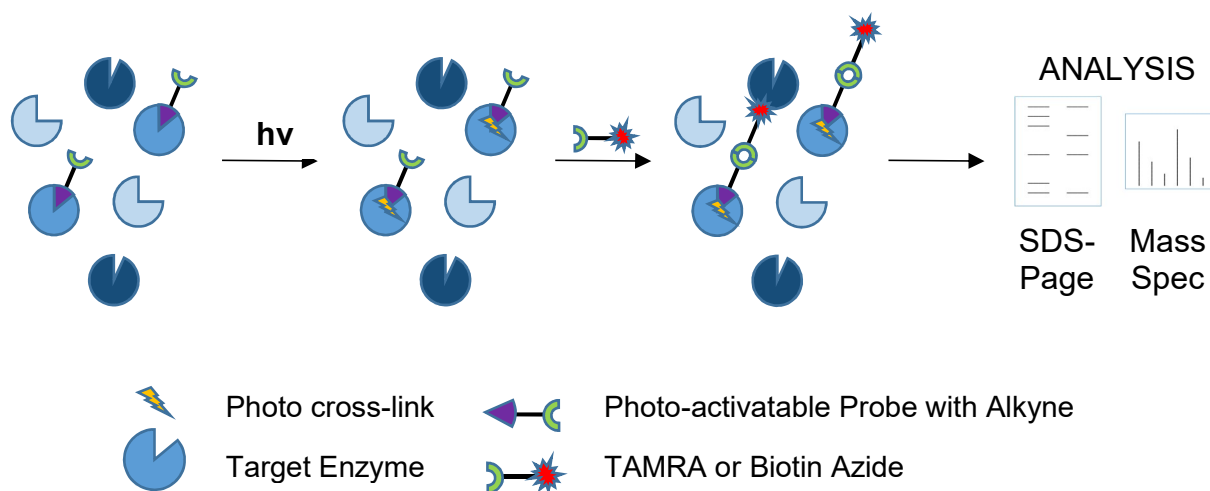


Figure 19: Schematic showing target labelling with a photoactivatable probe. Once the probe is bound to the target protein, UV irradiation causes the probe to react and form an irreversible covalent crosslink. A capture reagent can then be used for analysis.

Photo-crosslinkable probes have emerged as a powerful tool in chemical biology. Photo-crosslinking groups have been successfully incorporated into an antiviral compound for HIV (human immunodeficiency virus),¹⁴² into unnatural nucleosides to make mRNA probes,¹⁴³ into phospholipids to identify binding partners,¹⁴⁴ and into a clinical candidate for cancer therapy, to identify its mechanism of action.¹⁴⁵ In the context of epigenetics, photo-crosslinkable probes have been developed to study HDACs. For instance, the photoactivatable probe SAHA-Bpyne (Figure 20) based on the HDAC inhibitor Vorinostat (Figure 11), was designed to bind to the enzyme active site in a complex proteome, allowing for HDAC targets to be identified, and differences in HDAC content in disease cells analysed.¹⁴⁶

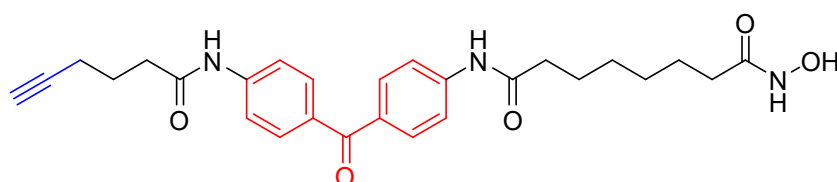


Figure 20: Chemical structure of SAHA-Bpyne, a photo-activatable probe based on the structure of HDAC inhibitor Vorinostat (or SAHA) containing a benzophenone as the photo-crosslinking group (red) and an alkyne handle (blue) for click chemistry with a capture reagent. Designed by Salisbary *et al.*¹⁴⁶

1.4.3. Mass Spectrometry Proteomics

A vital part of these techniques is the ability to identify the proteins, once they have been labelled by the probe containing the proper reporter group or coupled to it through click chemistry. Mass spectrometry (MS) is therefore an invaluable tool in proteomics, allowing for protein characterisation of large complex samples.

There are two main strategies for protein characterisation. The first is a 'top-down' approach, where intact proteins are analysed, although this is mainly effective for smaller, purified proteins, rather than complex mixtures requiring separation.¹⁴⁷⁻¹⁴⁸

The main technique used for target identification of a given inhibitor in the proteome of interest is 'bottom-up' or 'shotgun' proteomics, where proteins are broken down into peptides for identification,¹⁴⁸⁻¹⁵² after being affinity enriched. The work-flow for this approach is shown in Figure 21. In this approach affinity for a particular molecule is used to enrich this complex proteome for the targets of the molecule. A common method is to use the strong biotin-avidin interaction to pull-down the biotin-containing targets with avidin, NeutrAvidin or streptavidin beads.

After enrichment, proteins are digested into smaller peptides for injection into the mass spectrometer. This process involves reduction of disulfide bonds and alkylation to prevent disulfide bonds reforming, which helps proteins unfold and facilitates cleavage during digestion, most commonly carried out with trypsin.¹⁵³

The peptides are then separated by HPLC (high-performance liquid chromatography) and ionized using a soft-ionization technique to provide information of peak intensities vs m/z (mass-to-charge ratios). Parent ions obtained from this first MS scan are then selected for further fragmentation using a higher energy ionisation mode, to provide a tandem MS of the fragments. As peptides fragment in a predictable way, analysis of the obtained fragments using specifically designed algorithms allows for peptide identification.¹⁵⁴⁻¹⁵⁸ Identification of the peptides allows for identification of the proteins by searching the proteome.

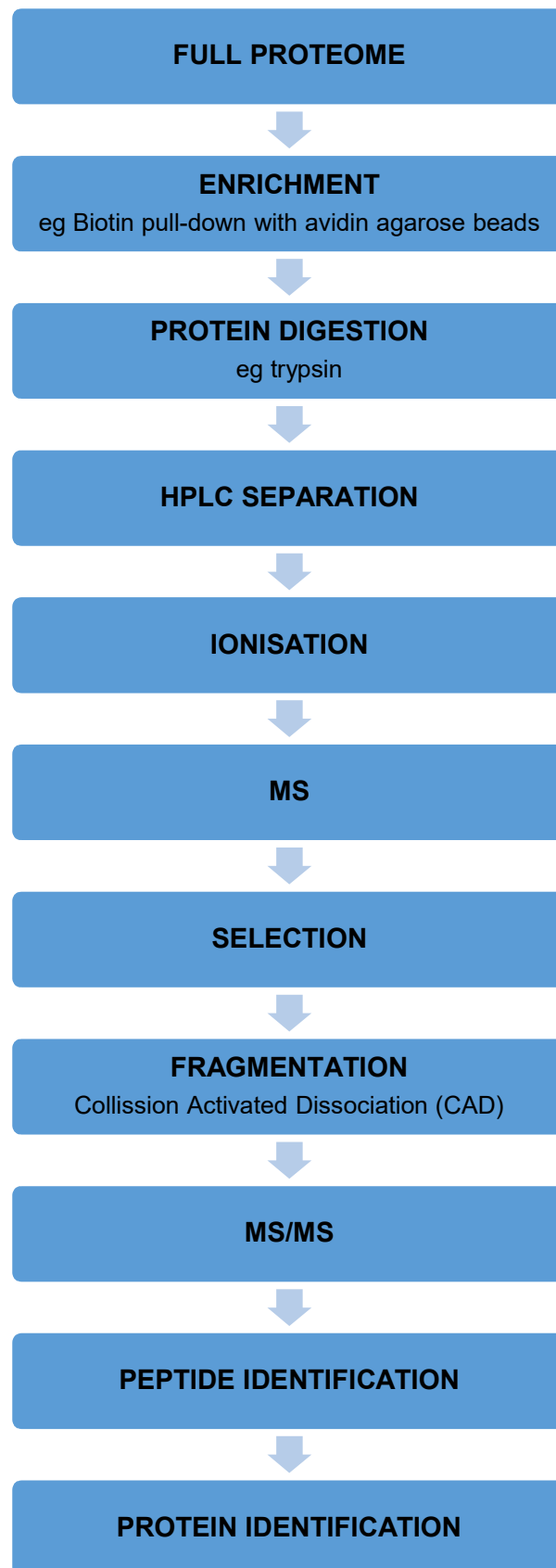


Figure 21: Work-flow for shotgun proteomics to identify proteins from a large sample using tandem mass spectrometry.

1.4.4. Examples of Probes for Malaria

Proteomics have been successfully used to identify targets of compounds with antimalarial activity. Activity probes have been used to probe the mode of action of artemisinin. Ismail *et al.* synthesised artemisinin derivatives designed to maintain activity whilst including an alkyne handle for click-chemistry and biotin for pull-down (Figure 22A).¹⁵⁹ An active artemisinin derivative and a non-peroxide inactive control were synthesised. They identified 58 proteins labelled by the active probe (Figure 22B), including potential targets from a number of essential pathways in *Plasmodium* including glycolysis, haemoglobin degradation and protein synthesis.

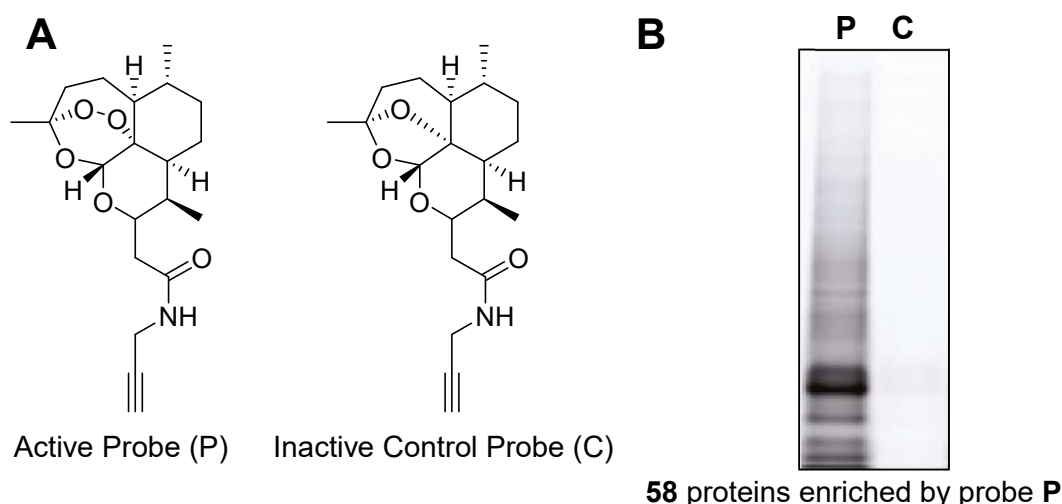


Figure 22: A) Chemical structures of the artemisinin-based activity probe **P** and the inactive control **C**. B) SDS-PAGE showing proteins labelled by active probe **P**. 58 proteins were enriched by probe **P** compared to the control **C**. Fluorescence is shown in grayscale. Work and figure by Ismail *et al.*¹⁵⁹

Photo-crosslinkable probes have also been used for target identification in malaria. Albitiazolium (Figure 23A) is an antimalarial compound with low nanomolar potency, that entered phase II clinical trials. It was designed to inhibit phospholipid metabolism in *Plasmodium*, a pathway essential to parasite survival.¹⁶⁰⁻¹⁶²

Penarete-Vargas *et al.* hypothesised that albitiazolium may have multiple targets in the parasite, and so designed a chemical probe with the aim of identifying these targets.¹⁶³

Compound UA1936 (Figure 23B) was designed as a bifunctional probe, containing a photo-crosslinkable aryl azide (shown in red), and a benzylic azide (shown in blue) for click chemistry with capture reagents. The probe had a very similar potency to the original molecule: 4.5 nM compared with 4.2 nM. Incubation of the probe with living parasites followed by click chemistry with fluorophore Alexa647 showed a number of labelled proteins using in-gel fluorescence. By culturing erythrocytes with the probe and performing in-cell click chemistry, localisation of the probe in the parasite cytosol could be observed at different life-cycle stages. Pull-down with alkyne-agarose resin followed by protein digestion allowed the identification of eleven interacting proteins including a putative phosphotransferase involved in phospholipid metabolism. In competition experiments with albitiazolium, the probe could be out-competed for this protein, suggesting this is a specific target and supporting the proposed mechanism of action. These examples show that this is a valid approach for target identification in malaria.

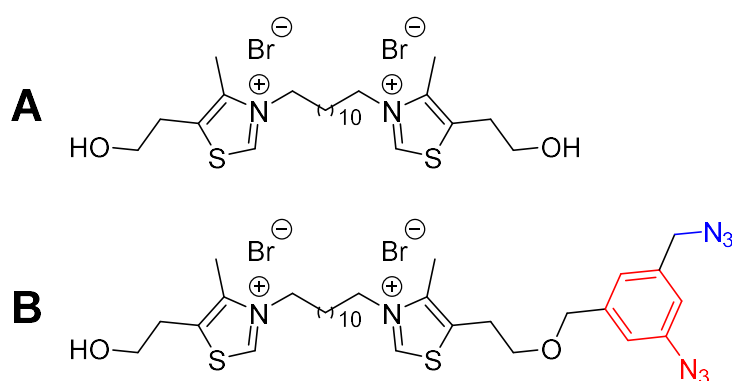


Figure 23: A) Albitiazolium, a bifunctional bis-thiazolium derivative with potent antimalarial properties. B) UA1936: a bifunctional probe based on the structure of albitiazolium, with an aryl azide for photo-crosslinking (red) and a benzylic azide for click chemistry (blue). Designed by Penarete-Vargas *et al.*¹⁶³

1.5. Project Aims

BIX-01294 and analogues present exciting antimalarial activity, with potent antimalarial activity at all blood-stages, and exciting *in vivo* activity. There is some indirect evidence for HKMT inhibition by these compounds, but in the absence of expressed and purified *Pf*HKMTs, further target identification and validation are needed. Photo-crosslinking probes and proteomics offer a powerful method for target identification where recombinant proteins are not available. Hence this project will focus on the design and development of photo-crosslinking probes for the validation of HKMTs as new therapeutic targets in *P. falciparum*.

2. Results and Discussion Part I: Development of the SAR

The structure-activity relationship, or SAR, of a compound series is the relationship between the structure of a molecule and its biological activity. It describes how changes to the chemical structure affect the potency and is vital for drug-discovery. It allows for the development of more potent and selective compounds and can also provide information about the binding site of the molecule. For the design of photo-crosslinking probes, a well-developed SAR is vital, as they need to maintain the biological activity of the original molecules.

2.1. Background

Previous work synthesising a library of compounds based on the BIX-01294 diaminoquinazoline series has allowed the development of a detailed SAR for this scaffold against *P. falciparum* and, crucially, highlighted opportunities to gain selectivity for the parasite over the human HKMT G9a and vice versa.^{103,164} The main explored positions prior to the start of this work are marked in different colours in Figure 24A.

A number of different substituents can be tolerated in position 2 (blue, Figure 24A), as illustrated by structures B-D and G in Figure 24. A variety of ring sizes are tolerated, and removal of the second nitrogen or introduction of a methoxy group maintain parasite killing activity.^{103,164} Interestingly, when a pyridine substituent is introduced to the piperazine ring (Figure 24C), the compound is no longer active against G9a, allowing for parasite selectivity. A primary acyclic amine in this position, however, abolishes activity.¹⁶⁴ These results indicate that this part of the molecule is likely accommodated in a big pocket in the target(s), and doesn't provide additional interactions. The number of groups tolerated in this position makes it a suitable point for diversification.

In position 4, the primary amine is essential to activity (green arrow, Figure 24A) - replacement of this functionality by an *N*-methyl, oxygen or sulfur leads to a large drop in activity (5-80 fold), suggesting this amine makes an important interaction in the binding pocket(s).¹⁰³ A variety of groups are tolerated on the piperidine ring (red, Figure 24A), including methyl (Figure 24C) and benzyl (Figure 24 and BIX-01294) allowing for diversification at this position. However the introduction of acyl groups into this position is not tolerated.^{103,164} These findings suggest that hydrophobic interactions may be important in this position, or the basicity of the nitrogen, which is much higher for an amine compared to an amide, and so may be protonated at physiological pH.

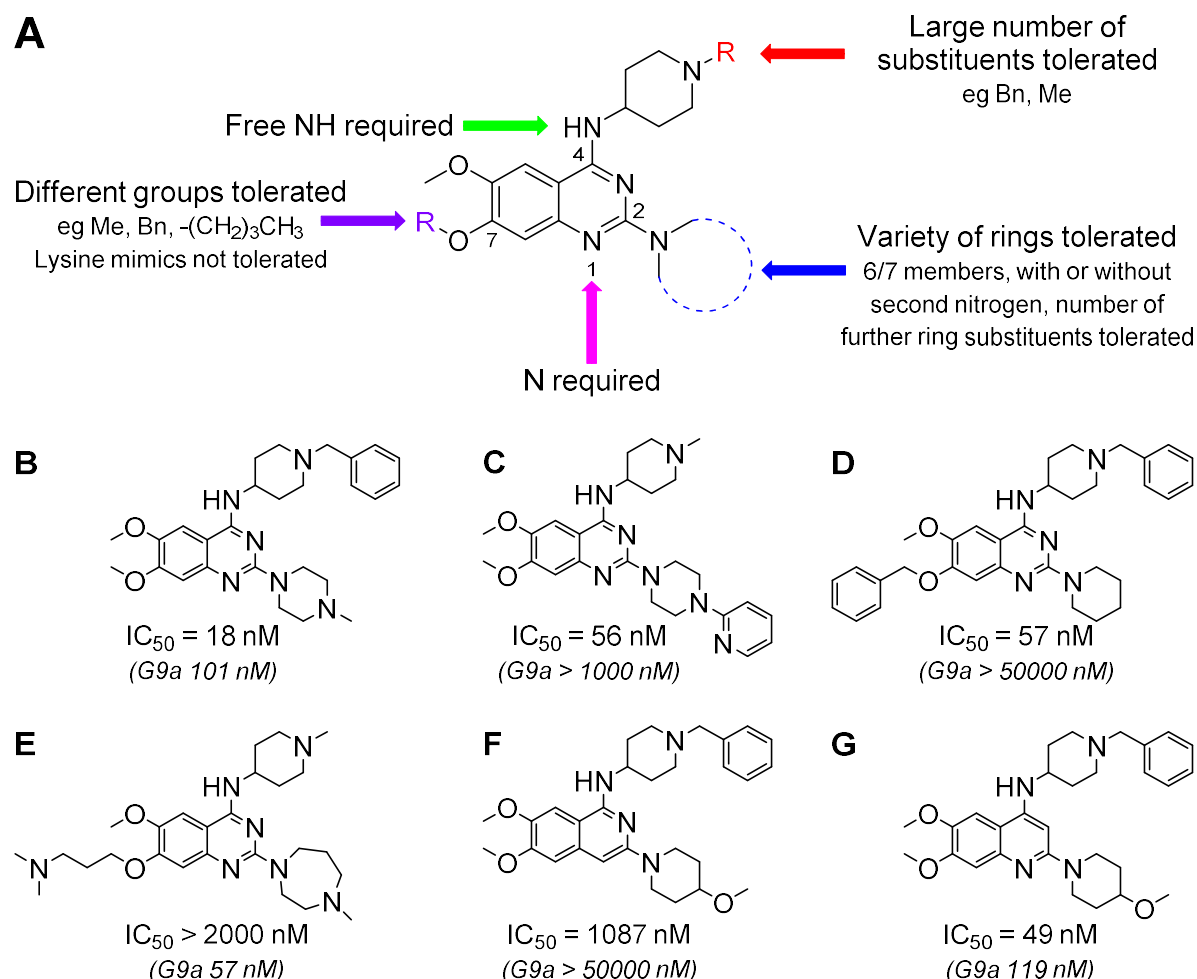


Figure 24: A) The SAR of diaminoquinazolines for parasite-killing activity.^{103,164} The quinazoline core, N-H at position 4, and basic nitrogen at position 2 are all essential for activity. Several substituents can be tolerated on the piperidyl ring at position 4, a number of rings with or without substituents can be introduced at position 2, and there is also tolerance of some substituents at position 7. B-G) Representative chemical structures from the library of compounds previously synthesised to determine the SAR, and their anti-*Plasmodium* activity.

Substitution of the oxygen in position 7 (purple, Figure 24A) allows for diversification and selectivity. While the introduction of a benzyl group affords for selectivity for the parasite over G9a (Figure 24D),^{103,164} the introduction of a lysine mimic (an amine-containing carbon chain) has been found to increase potency against G9a instead (Figure 24E).¹⁶⁵⁻¹⁷¹ The lysine mimic sits in the lysine binding site in the active site of G9a, so the lack of activity of these mimics in *P. falciparum* may suggest that the structure of the target(s) differs in the parasite, with hydrophobic interactions being more important, or this may be a physicochemical effect. Hence position 7 may provide a valuable opportunity to gain selectivity.

In the quinazoline scaffold the nitrogen in position 1 (pink arrow, Figure 24A) is essential to potent activity, whereas the nitrogen in position 3 is not (Figure 24, F vs G).

In summary, the established SAR prior to the start of this project highlights the points available for diversification of the scaffold in order to get compounds with enhanced activity and selectivity as future anti-malarial therapeutics. Whilst the differences in the SAR between parasite killing and G9a inhibition are important for drug discovery efforts, the points of conservation between both suggest these compounds may be targeting similar SET domain(s) in *Plasmodium*. Indeed, the co-crystal structure of G9a with a similar inhibitor shows the nitrogen in position 1 (pink, Figure 24A), the free NH in position 4 (green, Figure 24A), and the nitrogen in the rings in positions 2 and 4 (blue and red, Figure 24A) are protonated and form hydrogen bonds with the substrate pocket of the SET domain in G9a.¹⁶⁹ Since all of these features are also needed for parasite activity, it can be rationalised that the binding site of the protein accountable for this activity in the parasite may be similar to that of G9a. Since the SET domains in the HKMTs are highly conserved across species,⁶⁷ it is reasonable to think that these compounds are binding to one or more *P. falciparum* HKMTs, although further evidence is needed.

2.2. Expanding the SAR: Improving Pharmacodynamic Properties

2.2.1. Rationale

Whilst this compound series have potent antimalarial properties, a number of other factors are important for drug discovery and lead compound development. In order to move this compound series forward, pharmacodynamic and pharmacokinetic properties will need to be improved, for example their metabolism and absorption, whilst maintaining or improving the potency and selectivity for *Plasmodium falciparum*.

One important parameter in drug discovery is $\text{Log}P$. The partition co-efficient P is the ratio of solubility in hydrophobic octanol and water, and so $\text{Log}P$ provides a measure of lipophilicity. Lipophilicity is important for hydrophobic binding to the target molecule, and influences the ADMET (absorption, distribution, metabolism, excretion and toxicity) properties. A higher lipophilicity will increase cell permeability and absorption but decrease solubility and metabolic stability. Analysis by GlaxoSmithKline (GSK) led to the development of the GSK 4/400 rule, which suggests that a molecular weight of less than 400, and a $\text{Log}P$ value of less than 4 is optimum for a drug-like molecule and reduces the risk of poor ADMET properties.¹⁷² *In vivo* toxicity data even suggests that $\text{Log}P$ of less than 3 increases the chance of tolerability.¹⁷³

Liu *et al* have developed the diaminoquinazoline series as G9a inhibitors, and have successfully developed compounds with favourable *in vivo* properties.¹⁶⁷⁻¹⁶⁸ Whilst in position 7 they use a lysine mimic, which the SAR shows us is not tolerated for anti-*Plasmodium* cytotoxicity,^{103,164} their approach in positions 2 and 4 is consistent with the SAR and so could be applied to this series as antimalarials. For example, the *N*-benzyl and *N*-methyl groups in the examples in Figure 24 are susceptible to oxidation, and so reduce the metabolic stability of the molecules *in vivo*. Blocking the sites of oxidation or removing these groups could therefore increase the metabolic stability of these molecules, and hence improve their pharmacodynamic properties.¹⁷⁴ Decreasing $\text{Log}P$ has been linked to an increase in

metabolic stability. BIX-01294 has a calculated Log P value of 3.82, which can therefore be optimised.

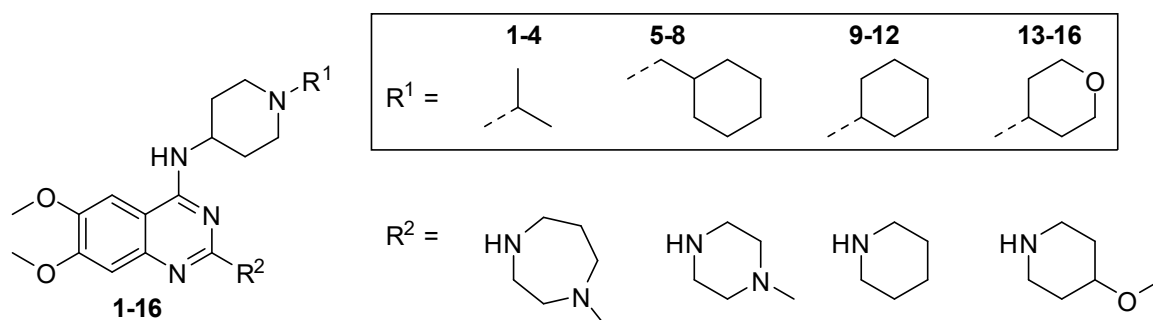
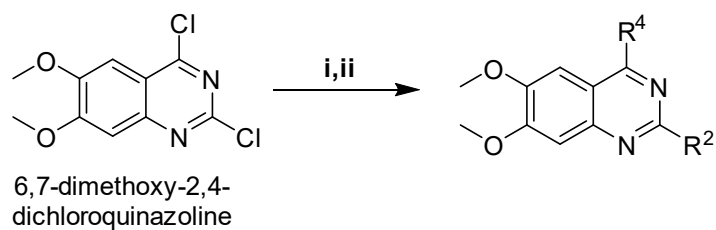


Figure 25: Groups to be introduced for the development of the SAR in position 4, with the aim of improving pharmacodynamic properties. A number of tolerated amines will be used in position 2.

Hence, a number of *N*-substituted-4-piperidylamines will be introduced in position 4, as shown in Figure 25. A number of groups have been tolerated in position 4, and it is not yet a position that has been found to give particular selectivity for either *Plasmodium* cytotoxicity or G9a,^{103,164} making it a good position for this diversification, which will allow selectivity to be built in elsewhere. These groups have been tested in similar molecules against G9a to improve metabolic stability and *in vivo* properties whilst maintaining activity.¹⁶⁷⁻¹⁶⁸ In position 2 a number of amines from the active compounds (Figure 24) will be introduced to make a small library of compounds **1-16**.

2.2.2. General Synthesis of Diaminoquinazolines

The chemical synthesis of these diaminoquinazolines is straightforward, as shown in Scheme 1. The methodology is well-established,^{103,166-167,169,175} and exploits the difference in reactivity of positions 2 and 4 on the dichloro-quinazoline scaffold for nucleophilic aromatic substitution by nucleophiles.

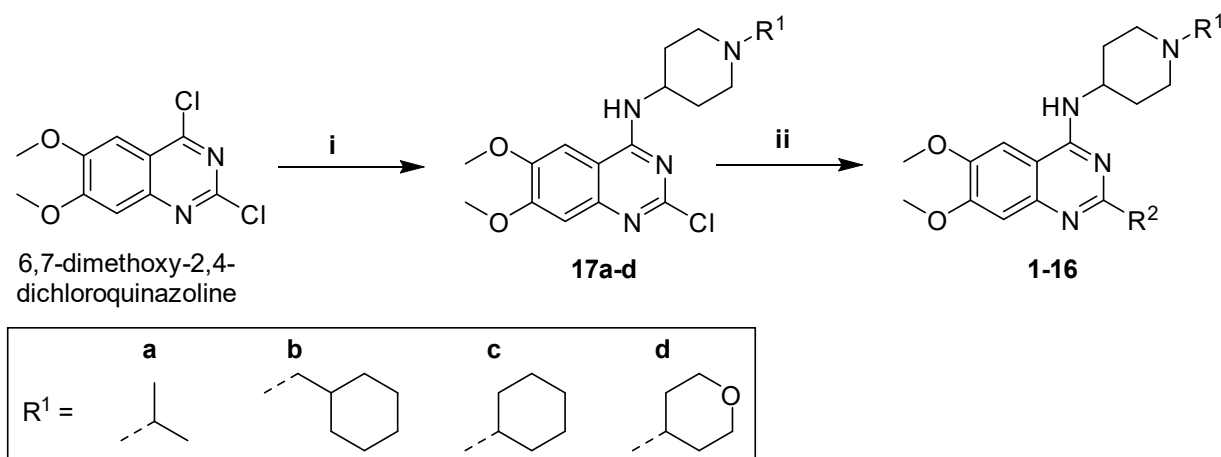


Scheme 1: The synthesis of diaminoquinazolines: i) various amines, DIPEA, THF, 30 °C, 18-24 hours; ii) various amines, toluene, microwave, 130 °C, 1-2 hours.

Hence 6,7-dimethoxy-2,4-dichloroquinazoline is first reacted with an *N*-substituted-4-piperidylamine to obtain the 4-substituted quinazoline. This can then be further reacted with the desired position 2 amine. Although these steps are simple, the final compounds can be difficult to purify which can reduce yields. This simple step-wise synthesis allows for fast diversification of the library.

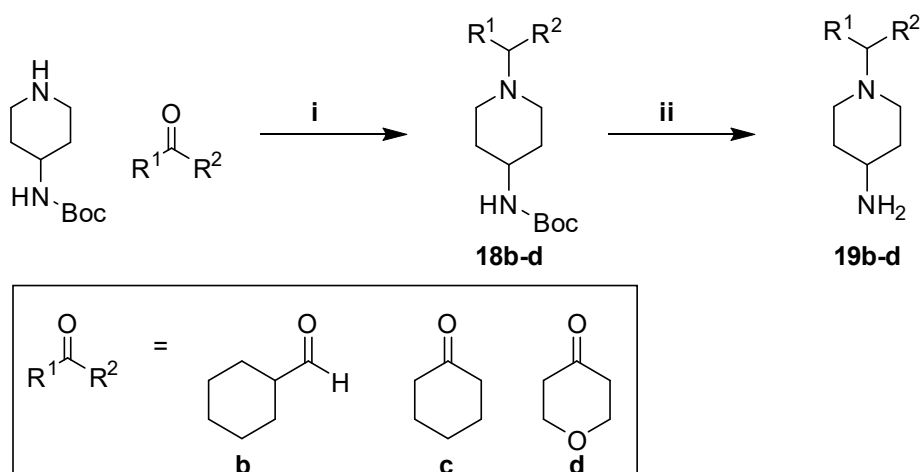
2.2.3. Synthesis

The synthesis of compounds **1-16** was carried out using the simple two-step synthesis described above, as shown in Scheme 2.



Scheme 2: The synthesis of diaminoquinazolines **1-16**: i) various *N*-substituted-4-piperidylamines **a-d**, DIPEA, THF, 30 °C, 18-24 hours, 24-48%; ii) R₂ amines, toluene, microwave, 130 °C, 1-2 hours, 14-78%.

Where the amines for position 4 were not commercially available, they were synthesised to give compounds **19b-d** as shown in Scheme 3.



Scheme 3: Synthesis of amines by reductive amination and Boc deprotection: i) $\text{NaBH}(\text{OAc})_3$, 1,2-dichloroethane, rt, 16 hours, 49-96%; ii) TFA, DCM, 4 hours, 51-97%.

Reductive amination with of 4-(N-Boc-amino)-piperidine with the necessary aldehyde or ketone using sodium triacetoxyborohydride¹⁷⁶ provides the Boc-protected amine with yields of 49-96%. This is followed by Boc deprotection with trifluoroacetic acid (TFA).

Hence, a small library of compounds **1-16** was synthesised.

2.2.4. Results

To assess the antimalarial activity of these compounds, a 3-day SYBR Green I assay was carried out by the Pasteur Institute in Paris to assess parasite growth after treatment with various concentrations of the compounds to determine the IC_{50} .¹⁷⁷ For comparison, the G9a inhibition of some of these compounds was also measured in a G9a biochemical assay by the Structural Genomics Consortium in Toronto.¹⁶⁸ The results are shown in Table 4.

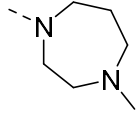
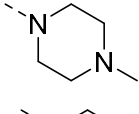
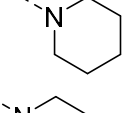
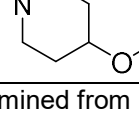
All of the groups introduced in position 4 were tolerated for antimalarial activity with comparable potency, although there is a small loss of activity in most cases. Differences in

potency between all the analogues, and any selectivity for *Plasmodium* or G9a, was more influenced by the choice of amine for position 2 than the novel position 4 amine. The piperidine and 4-methoxy-piperidine generally show improved potency and selectivity for parasite killing activity. Compounds **12** and **15** were the most potent, with IC₅₀ values showing a small improvement compared with BIX-01294. These compounds have more favourable Log*P* values compared to BIX-01294 (3.56 and 2.75 compared with 3.82), and so may show improved metabolic stability and other *in vivo* physiochemical properties. The compounds with the lowest Log*P*, for example compounds **13** and **14** (1.88 and 1.77), however, may start to present problems with absorption in an *in vivo* model. These compounds have added to a large library of diaminoquinazolines, which has allowed for the publication of the SAR of this series for G9a and *P. falciparum*, and the identification of both the similarities and the opportunities for selectivity.¹⁶⁴

The pharmacochemical properties of these compounds have not yet been tested, but these results suggest that the benzyl group from BIX-01294 could be replaced with a lipophilic and potentially less labile group, without loss of activity.

Table 4: *Plasmodium falciparum* cytotoxicity and G9a inhibition for compounds designed to expand the SAR of position 4 of the diaminoquinazolines, to improve the pharmacodynamic properties. Anti-plasmodium activity was measured by the Pasteur Institute in Paris. G9a activity was measured by the Structural Genomics Consortium in Toronto. Values are given as mean $\pm$ SD.

	R ¹	R ²	<i>Pf</i> 3D7 IC ₅₀ [*] (nM)	G9a IC ₅₀ [*] (nM)	G9a / <i>Pf</i> 3D7	cLogP ⁺	TPSA ⁺
	<i>BIX-01294</i>		43	67	1.6	3.82	64.9
1			470 ^a $\pm$ 221	55 ^a $\pm$ 8	0.12	2.74	64.9
2			280 ^a	123 ^a $\pm$ 18	0.44	2.63	64.9
3			71 ^a $\pm$ 19	319 ^a $\pm$ 47	4.5	3.61	61.7
4			126 ^a $\pm$ 50	NT	-	2.67	70.9
5			174 ^a $\pm$ 63	10 ^a $\pm$ 1	0.06	4.06	64.9
6			130 ^a $\pm$ 62	25 ^a $\pm$ 3	0.19	3.95	64.9
7			144 ^a $\pm$ 88	295 ^a $\pm$ 33	2.0	4.93	61.7
8			47 ^a $\pm$ 24	185 ^a $\pm$ 24	3.9	3.99	70.9
9			205 ^a	55 ^a $\pm$ 7	0.27	3.63	64.9
10			222 ^a $\pm$ 125	49 ^a $\pm$ 13	0.22	3.53	64.9
11			108 ^a $\pm$ 52	NT	-	4.50	61.7
12			28 $\pm$ 5	NT	-	3.56	70.9

13		447 ± 201	NT	-	1.88	74.2
14		179 ± 39	NT	-	1.77	74.2
15		28 ± 5	NT	-	2.75	70.9
16		55 ± 9	NT	-	1.81	80.2

* IC₅₀ mean value determined from duplicates or triplicates.

NT = Not Tested.

^a published data¹⁶⁴

⁺ TPSA as predicted by ChemDraw Professional 16.0.

2.3. Expanding the SAR: Investigating Aromatic Substituents

2.3.1. Rationale

The benzyl group of BIX-01294 offers an additional point for diversification and the potential to gain potency or selectivity. Previously, methyl, methoxy and fluorine aromatic substituents have been tested and tolerated, maintaining similar parasite-killing as well as increasing the selectivity over G9a.^{103,164} However, no electron-withdrawing substituents have been tested.

Hence, ester, nitrile and amide analogues were designed, as illustrated in Figure 26. Para substituents were used to minimise steric interference during synthesis. These substituents will also have an effect on the topological polar surface area (TPSA) of the compounds, without any significant alterations to other physicochemical properties such as Log*P*, allowing the effect of TPSA to be assessed. The introduction of polar groups can in some instances aid permeability and binding interactions, however high TPSA can reduce the ability of molecules to permeate cell barriers, and hence can reduce cellular uptake. The Pfizer 3/75 rule suggests a Log*P* of less than 3, and a TPSA of greater than 75 is optimal for reducing toxicity risk of drug-like compounds, and improving *in vivo* properties.¹⁷³

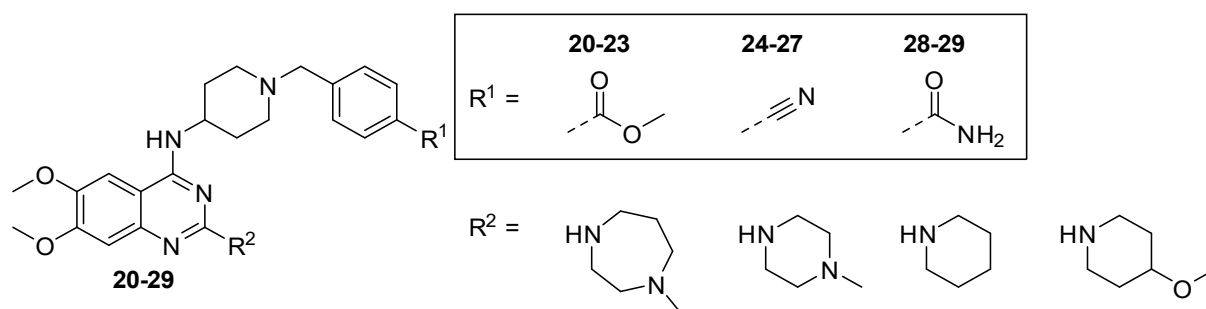
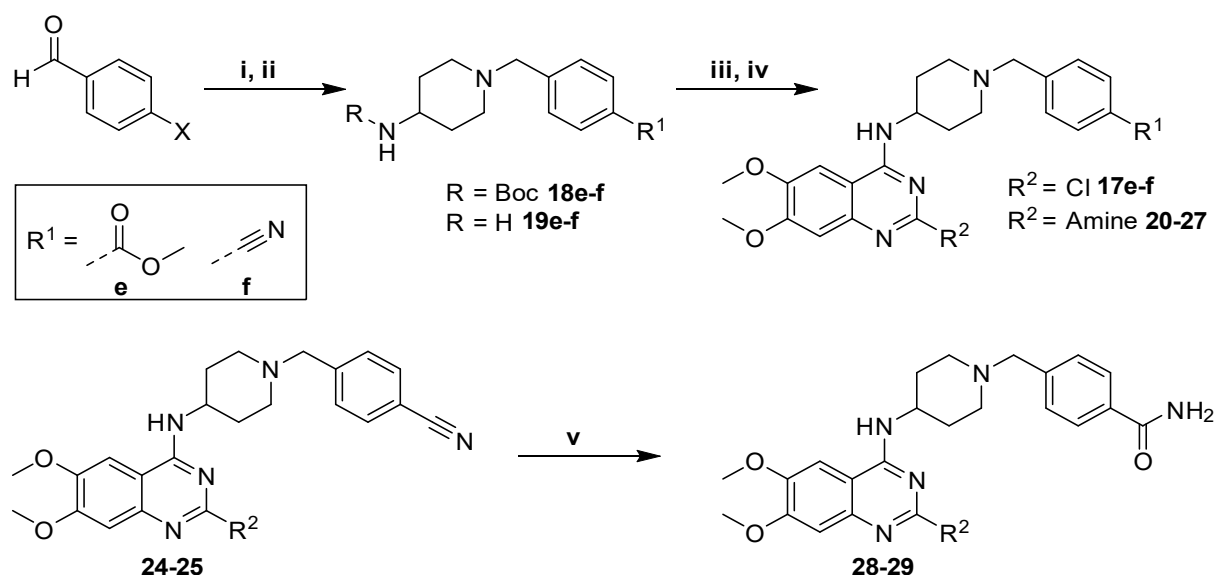


Figure 26: Groups to be introduced for the development of the SAR in position 4, to explore the effect of substituents of the benzene ring. A number of tolerated amines will be used in position 2.

2.3.2. Synthesis

As previously, these compounds were synthesised using simple two-step procedure (Scheme 1). The synthesis of these compounds is shown in Scheme 4. To synthesise the amines for position 4, ester and nitrile benzaldehyde derivatives were used in reductive amination reactions as before. This was followed by Boc deprotection, and the sequential reaction of 6,7-dimethoxy-2,4-dichloroquinazoline with the amines to yield final compounds **20-27**.



Scheme 4: Synthesis of compounds with benzene derivatives. i) NaBH(OAc)₃, 1,2-dichloroethane, rt, 16 hours, 74-76%; ii) TFA, DCM, 4 hours, 77-88%; iii) DIPEA, THF, 30 °C, 18-24 hours, 32-50%; iv) R₂ amines, toluene, microwave, 130 °C, 1-2 hours, 28-93%; v) K₂CO₃, H₂O, microwave, 150 °C, 1 hour, 17%.

For nitrile compounds **26** and **27**, which were produced in sufficient quantities for further reaction, the nitrile was hydrolysed to an amide using potassium carbonate and water under microwave irradiation,¹⁷⁸ to yield compounds **28** and **29**. This reaction was fairly poor yielding, with yields of around 17% but gave sufficient amounts of the pure products for biological testing, whilst avoiding the use of expensive catalysts and toxic solvents. A small library of compounds **20-29** was synthesised.

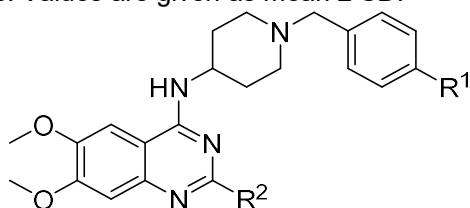
2.3.3. Results

As before, the antimalarial activity was measured by the Pasteur Institute, and the results are shown in Table 5, along with the calculated TPSA, which is significantly increased for all compounds compared with BIX-01294. Both the ester and nitrile groups are well tolerated, with comparable potency to BIX-01294 regardless of the amine in position 2. All of compounds **20-27** had IC₅₀ values of 24-63 nM, showing this to be a promising option for further compound development. The introduction of these groups also increases the TPSA to around 90, compared with 64.9 for BIX-01294, a more favourable value for drug-like compounds.¹⁷³

The amide on the other hand caused a loss of activity, with IC₅₀ values as high as 807 nM (**28**). The amide has similar electron withdrawing effects to the ester and nitrile, and has a similar effect on the TPSA, which may suggest these factors are not responsible for the observed effects, although the loss of activity could also be due to the solubility or cell permeability of the amide compared with the ester and nitrile.

These compounds have not been tested for G9a inhibition. This would be interesting, particularly the potent ester and nitrile compounds, to see if this offers any opportunity to gain selectivity.

Table 5: *Plasmodium falciparum* cytotoxicity for compounds designed to expand the SAR of position 4 of the diaminoquinazolines, to explore the effects of TPSA. Anti-*Plasmodium* activity was measured by the Pasteur Institute in Paris. Values are given as mean $\pm$ SD.



	R ¹	R ²	<i>Pf</i> 3D7 IC ₅₀ [*] (nM)	cLogP ⁺	TPSA ⁺
		<i>BIX-01294</i>	43	3.82	64.9
20			60 $\pm$ 20	3.64	91.2
21			36 $\pm$ 15	3.53	91.2
22			63 $\pm$ 20	4.51	88.0
23			27 $\pm$ 7	3.57	97.2
24			52 $\pm$ 14	3.85	88.7
25			42 $\pm$ 16	3.74	88.7
26			24 $\pm$ 4	4.72	85.5
27			29 $\pm$ 4	3.78	94.7
28			807 $\pm$ 222	2.72	108.0
29			205 $\pm$ 24	2.62	108.0

* IC₅₀ mean value determined from duplicates or triplicates.

⁺ TPSA as predicted by ChemDraw Professional 16.0.

2.4. Conclusions

These results have helped further develop the SAR, providing more information to develop potent and selective compounds for anti-*Plasmodium* activity, and will aid future compound development towards future therapeutics. A number of compounds including **12**, **15**, **23**, **26** and **27** showed potent activity, and may provide a starting point for future development, for example through the introduction of hydrophobic substituents onto the oxygen in position 7. These compounds have improved Log*P* and TPSA values, which follow the GSK 4/400 and Pfizer 3/75 rules, which guide the development of more drug-like lead compounds.¹⁷²⁻¹⁷³ Combining the substituents that provide the best potency and selectivity will allow for the synthesis of a more focused library for antimalarial activity. The pharmacodynamic and *in vivo* properties of this improved library can then be tested and developed, incorporating groups known to improve these properties in areas where they are tolerated. This further compound development will help lead to the discovery of a potent, selective *in vivo* inhibitor for anti-*Plasmodium* activity.

In addition, this SAR will allow for the development of photo-crosslinkable probes, allowing for target identification in an approach similar to those described in Chapter 1. The SAR will allow chemical tools to be developed, as the photo-crosslinking and alkyne moieties can be introduced in positions where it is known they will be tolerated, allowing the probes to maintain the biological activity of this compound series.

3. Results and Discussion Part II: Photo-crosslinkable Probes & Proteomics

As previously discussed, chemical proteomics¹²⁴⁻¹²⁷ and photo-crosslinkable probes¹⁴¹ have emerged as a powerful tool for target identification. These probes include an appropriate photoactive group which, when exposed to UV irradiation, allows for covalent linking of an inhibitor to its molecular targets. Incorporation of an alkyne handle allows for downstream target identification of the resulting protein-inhibitor complexes, to provide direct experimental evidence for targets of the probe. This approach was applied to the diaminoquinazoline series, to identify targets in *Plasmodium falciparum*, through the design and synthesis of photo-crosslinkable probes, followed by gel-based experiments and pull-down proteomics.

3.1. Probe Design and Synthesis

3.1.1. Probe Design

To synthesise photo-crosslinkable probes, a well-developed SAR is vital to introduce the photo cross-linkable group and alkyne handle, in order to ensure that the probes maintain the activity of the series and bind to the same targets. Among the different positions where the probe functionalities could be introduced (Figure 24A), positions 2 and 4 were chosen as the SAR shows a large number of groups are tolerated in this position, and it is synthetically straightforward. Thus, probes **1-4** were designed so that different groups and structures could be tested during the labelling protocol development, shown in Figure 27.

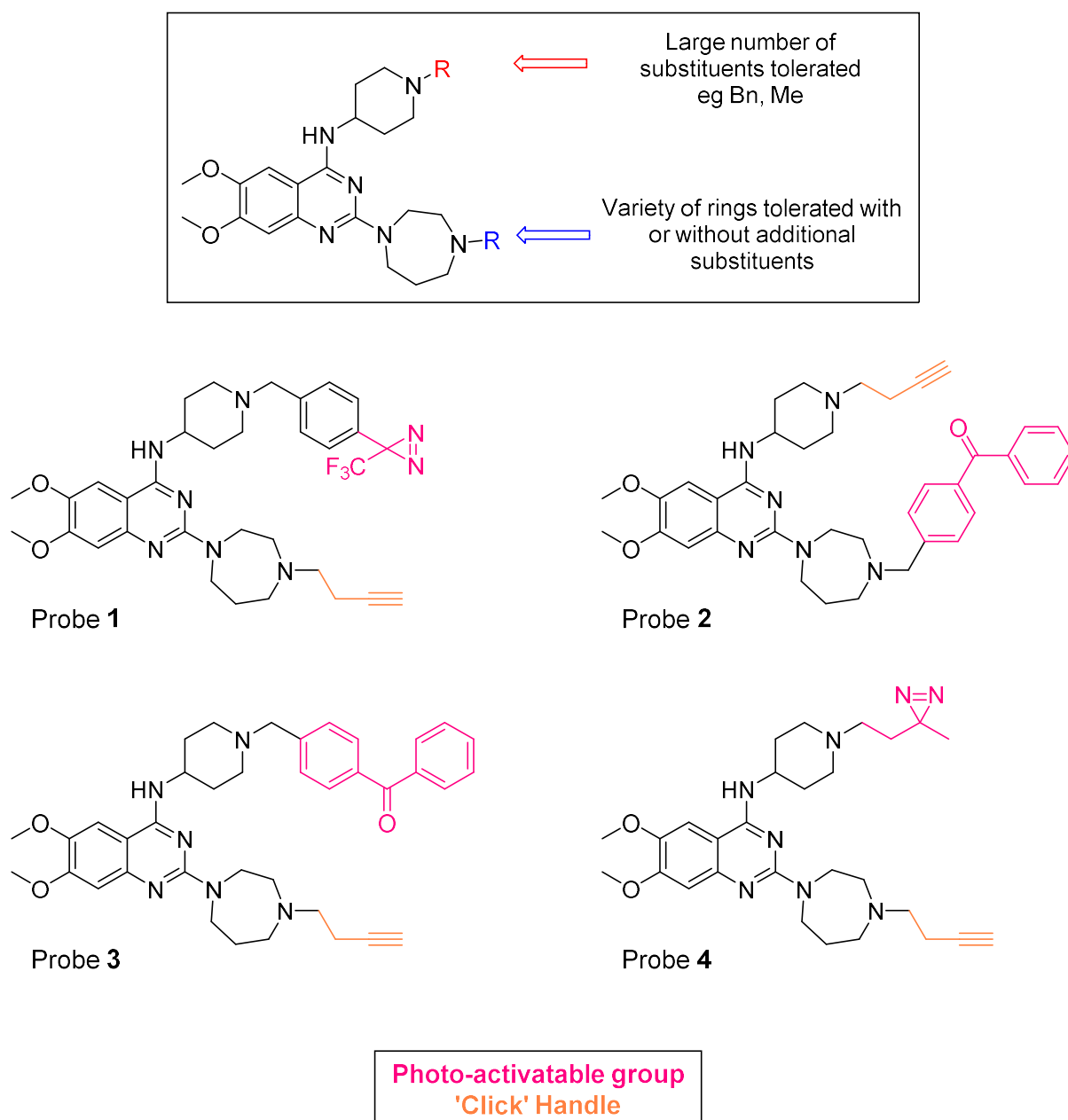


Figure 27: Design of photo-crosslinkable probes using the SAR of the diaminoquinazoline series: a photo-activatable group (diazirine or benzophenone, shown in pink) and an alkyne handle (shown in orange) were incorporated onto the piperidine ring in position 4 (red) or in position 2 (blue).

For the photo-activatable groups, diazirines and benzophenones were chosen. Diazirines are well established in photo-crosslinking methodologies, and their small size can minimise the impact on target-binding. Probe 1 incorporates a 3-trifluoromethyl-3-phenyldiazirine, which are popular choices as the reactive diazirine carbene does not rearrange internally

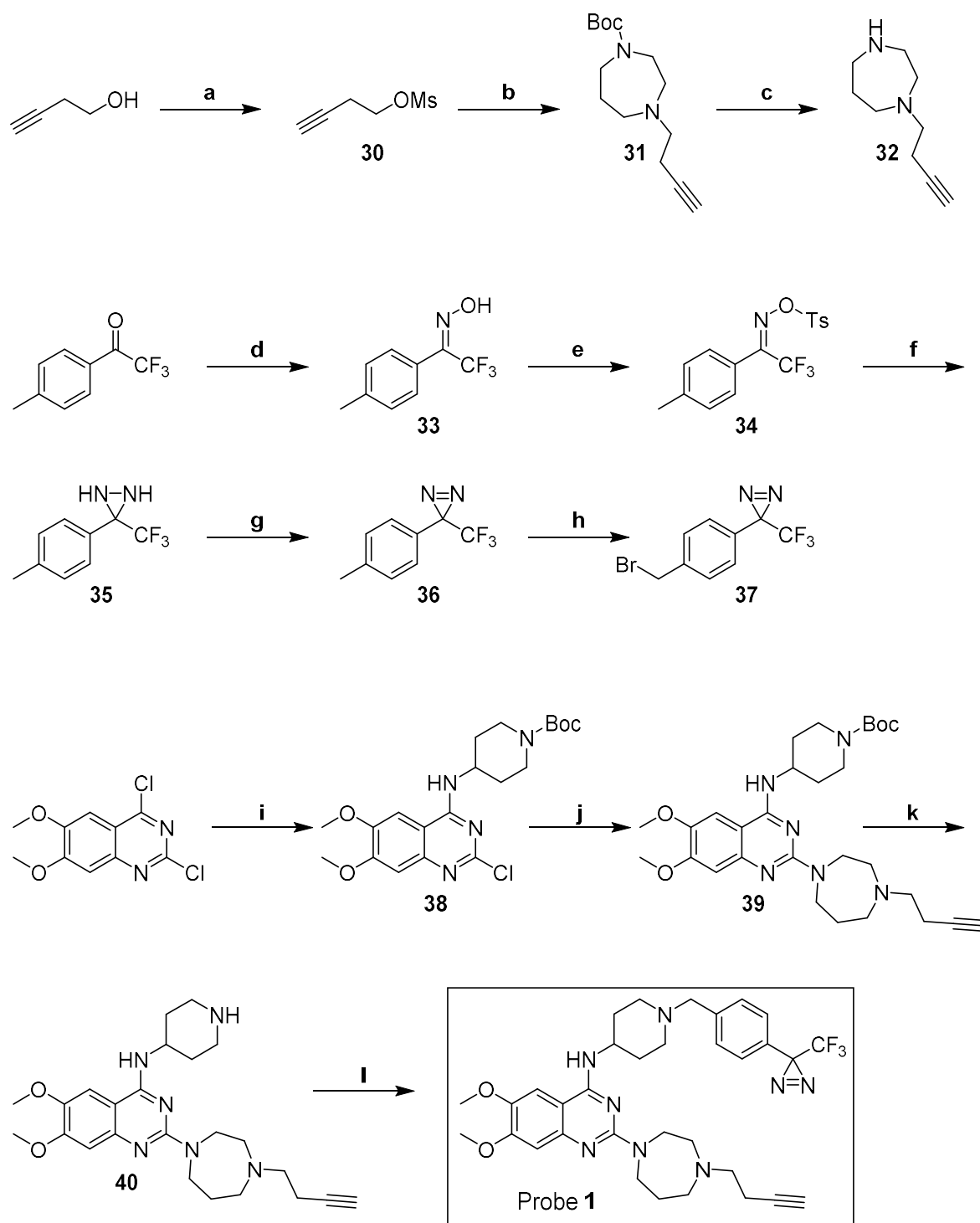
which can decrease photo-crosslinking yields.¹⁷⁹ Probe **4** incorporates an alkyl diazirine. Benzophenones are also well-established photo-crosslinkers, and were chosen due to their chemical stability and the fact that they are straightforward to introduce synthetically.¹⁸⁰ This group was introduced in two different positions in probes **2** and **3** for comparison. An alkyne handle was introduced into each probe, to allow for click chemistry with an azide-containing capture reagent.

3.1.2. Probe Synthesis

The synthesis of all four probes relies on the same methodology as the general synthesis of the diaminoquinazoline series, shown in Scheme 1 (Section 2.2.2) the dichloro-quinazoline scaffold is reacted step-wise with the desired functionalised amines.

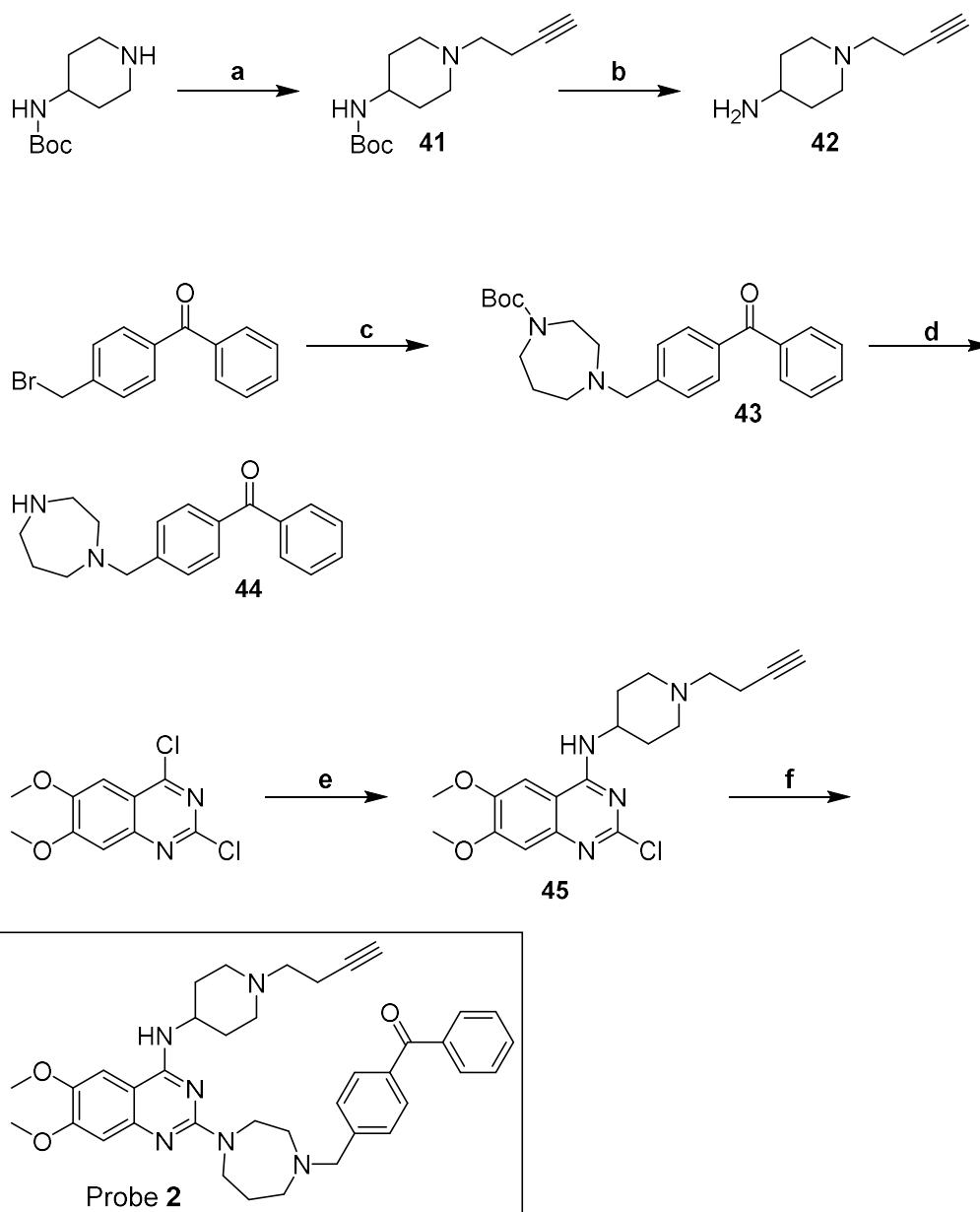
3.1.2.1. Probe 1

The synthesis of probe **1** required the synthesis of alkyne **32** and diazirine **37** (Scheme 5). Mesylation of 3-butyne-1-ol, followed by nucleophilic substitution with 1-Boc-hexahydro-1,4-diazepine and Boc deprotection provided amine **32** with a yield of 51% over 3 steps, which will also be used in the synthesis of probes **3** and **4**. The trifluoro-diazirine **37** was synthesised from 4'-methyl-2,2,2-trifluoroacetophenone following well-established procedures with liquid NH_3 .¹⁸¹⁻¹⁸² 4-Amino-1-Boc-piperidine and **32** were subsequently reacted with 6,7-dimethoxy-2,4-dichloroquinazoline as previously described to afford intermediate **39**, before final Boc deprotection. Reaction of the resulting amine with diazirine **37** yielded probe **1**.



Scheme 5: Synthesis of Probe 1: a) MsCl, NEt₃, DCM, rt, 16 h, 91%; b) 1-Boc-hexahydro-1,4-diazepine, K₂CO₃, NaI, MeCN, reflux, 3 h, 60%; c) TFA, DCM, rt, 4 h, 93%; d) NH₂OH.HCl, pyridine, reflux, 4 h, 93%; e) p-TsCl, NEt₃, DMAP, rt, 16 h, 71%; f) NH₃ (liq), Et₂O, -78 °C, 2 h, 83%; g) NEt₃, I₂, DCM, 0 °C, 1 h, 69%; h) NBS, AIBN, CCl₄, reflux, 1 h, 73%; i) 4-amino-1-Boc-piperidine, DIPEA, THF, 30 °C, 16 h, 17%; j) **32**, toluene, microwave, 130 °C, 1 h, 37%; k) TFA, DCM, rt, 4 h, 46%; l) **37**, K₂CO₃, MeOH, THF, rt, 16 h, 24%

3.1.2.2. Probe 2



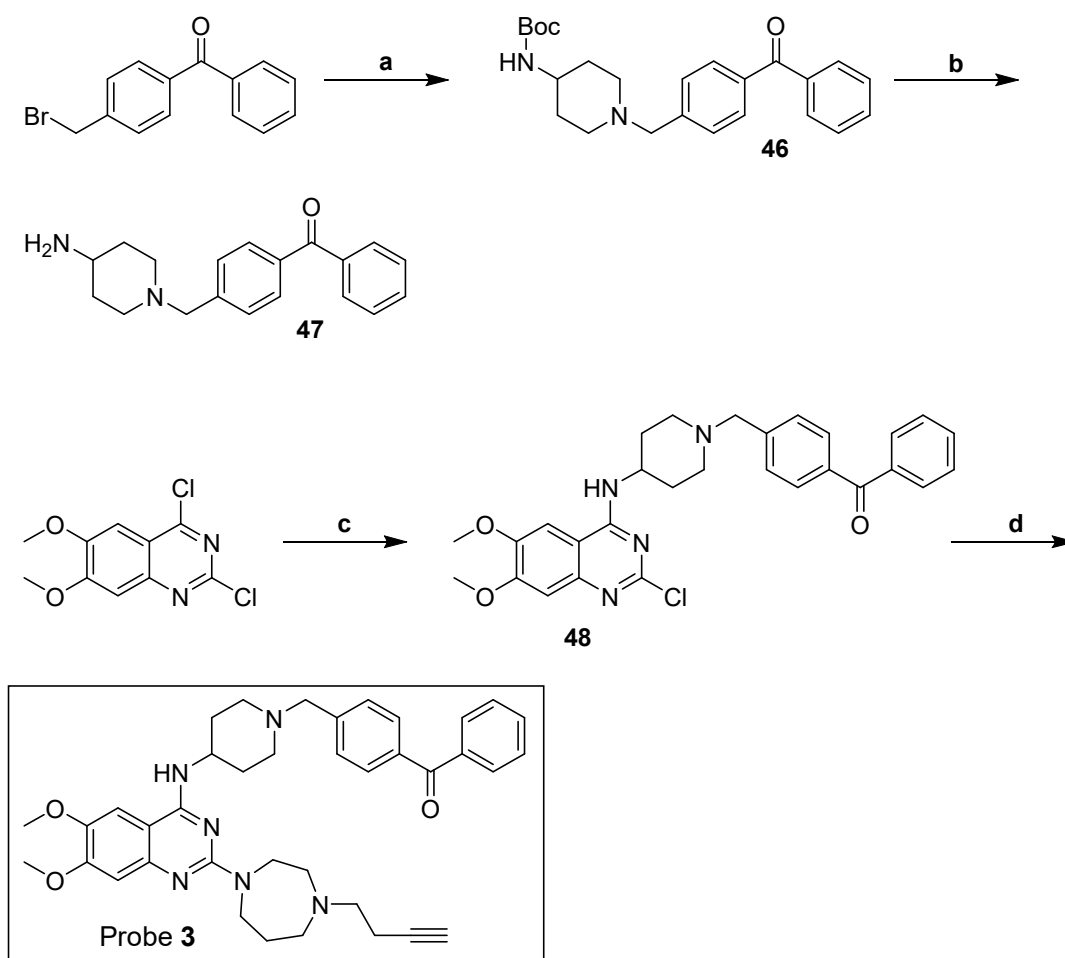
Scheme 6: Synthesis of Probe 2: a) **30**, NEt₃, KI, DMSO, rt, 16 h, 41%; b) TFA, DCM, rt, 4 h, 64%; c) 1-Boc-hexahydro-1,4-diazepine, NEt₃, DCM, rt, 24 h, 77%; d) TFA, DCM, rt, 4 h, 56%; e) **42**, DIPEA, THF, rt, 16 h, 34%; f) **44**, toluene, microwave, 130 °C, 1 h, 54%.

The synthesis of probe **2** is shown in Scheme 6. Alkyne **42** was prepared by nucleophilic substitution of **30** with 4-(*N*-Boc-amino)-piperidine followed by Boc deprotection. The synthesis of the benzophenone-containing subunit **44** started with the nucleophilic substitution of commercially available 4-(bromomethyl)-benzophenone with 1-Boc-

hexahydro-1,4-diazepine followed by Boc deprotection. Amines **42** and **44** could then be introduced to 6,7-dimethoxy-2,4-dichloroquinazoline respectively, to provide probe **2**.

3.1.2.3. Probe 3

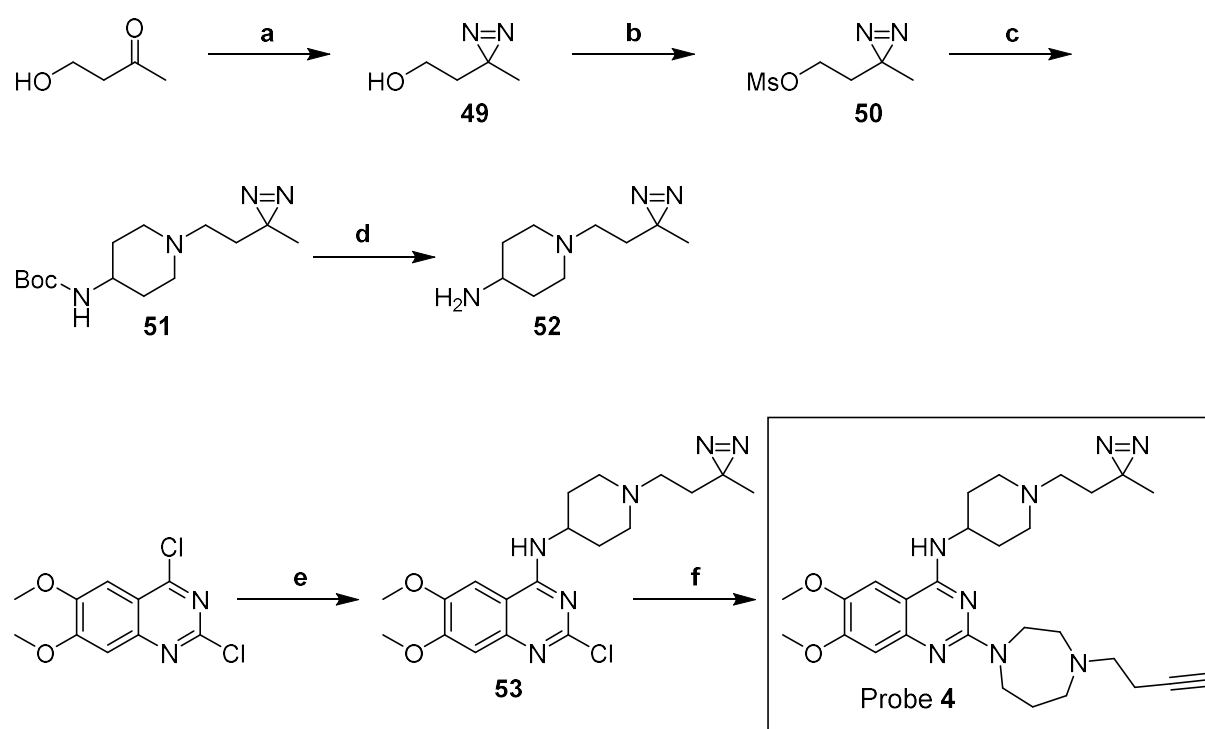
Probe **3** was synthesised using similar methodology to probe **2**, but to introduce alkyne and photo-activatable functionalities into the opposite positions in the final probe, as shown in Scheme 7. Amine **47** was prepared by nucleophilic substitution of 4-(bromomethyl)-benzophenone with 4-(*N*-Boc-amino)-piperidine followed by Boc deprotection of intermediate **46**. Finally, subsequent nucleophilic aromatic substitutions of 6,7-dimethoxy-2,4-dichloroquinazoline with amines **47** and **32** provided probe **3**.



Scheme 7: Synthesis of Probe **3**: a) 4-(*N*-Boc-amino)-piperidine, NEt₃, DCM, rt, 24 h, 76%; b) TFA, DCM, rt, 4 h, 78%; c) **47**, NEt₃, THF, rt, 16 h, 52%; d) **32**, toluene, microwave, 130 °C, 1 h, 24%.

3.1.2.4. Probe 4

The synthesis of probe **4** is shown in Scheme 8. Preparation of the alkyl diazirine moiety started from 4-hydroxy-2-butanone. Its reaction with liquid NH_3 , followed by the addition of hydroxylamine-O-sulfonic acid and oxidation of the resulting diaziridine with iodine followed previously described procedures.¹⁸³ Mesylation of the primary alcohol, followed by nucleophilic substitution with 4-(*N*-Boc-amino)-piperidine and Boc deprotection yielded amine **52**. Its reaction with 6,7-dimethoxy-2,4-dichloroquinazoline, followed by the introduction of amine **32** provided probe **4**.¹⁸⁴



Scheme 8: Synthesis of Probe **4**: a) i) $\text{NH}_3(\text{liq})$, $-78\text{ }^\circ\text{C}$, 4 h; ii) $\text{H}_2\text{NOSO}_3\text{H}$, MeOH, $-78\text{ }^\circ\text{C}$ – rt, 16 h; iii) NEt_3 , I_2 , MeOH, rt, 2 h, 46%; b) MsCl , NEt_3 , DCM, rt, 5 h, 87%; c) 4-(*N*-Boc-amino)-piperidine, K_2CO_3 , NaI, MeCN, reflux, 3 h, 65%; d) TFA, DCM, rt, 4 h, 95%; e) **52**, DIPEA, THF, rt, 16 h, 15%; f) **32**, toluene, reflux, 18 h, 4%.

3.1.3. Probe Antimalarial Activity

Following the synthesis of probes **1-4**, their antimalarial activity was measured by the Pasteur Institute in the 3-day SYBR Green I assay previously described, to ensure the probes maintain the biological activity of the inhibitor series. The results are shown in Table 6.

Table 6: *Plasmodium falciparum* cytotoxicity for synthesised probes. Values quoted as mean $\pm$ standard deviation over replicate experiments. Anti-*Plasmodium* activity measured by the Pasteur Institute in Paris.

Probe	IC ₅₀ (nM)
Probe 1	395 $\pm$ 179
Probe 2	128 $\pm$ 60
Probe 3	176 $\pm$ 82
Probe 4	42 $\pm$ 20

All probes maintained reasonable antimalarial activity, with a maximum IC₅₀ values ranging from 42-395 nM, compared to a value of 43 nM for BIX-01294. Probe **2** and Probe **4** were the most active, with IC₅₀ values of 128 nM and 42 nM respectively. These values suggest that probes **2** and **4** have best maintained the activity of the diaminoquinazoline compound series of interest, as expected from the SAR, and hence are suitable for use as photo-activatable probes for target ID for the series.

3.2. Lysate Labelling and In-Gel Fluorescence

Initially, the photo-crosslinking probes developed were used in gel-based experiments, to test the ability of the probes to label proteins, and to develop a labelling protocol. Briefly, the probes were added to *Plasmodium falciparum* lysate, photo-crosslinked and a fluorescent capture reagent conjugated by click-chemistry for visualisation.

3.2.1. Protocol Development

A protocol was needed to label the target proteins with the photo-crosslinkable probes in *P. falciparum*. Rather than using whole cells, cell lysate was chosen for these experiments, due to it being experimentally simpler to work with. Carrying out the experiment in whole-cells also requires the functionalised probe to cross the cell membrane and be stable to metabolism. The advantage of cell-based experiments is that they are more biologically relevant, and proteins will always be folded and functional, so these may be interesting experiments to carry out in the future.¹²⁵ For the lysate experiments, saponin-treated pellets derived from 30-40 hour post invasion, blood stage *P. falciparum* (3D7 strain) cultures were lysed by nitrogen cavitation.^{125,185} A protocol for lysate labelling was developed using previously reported methods,^{139,186-188} as depicted in Figure 28.

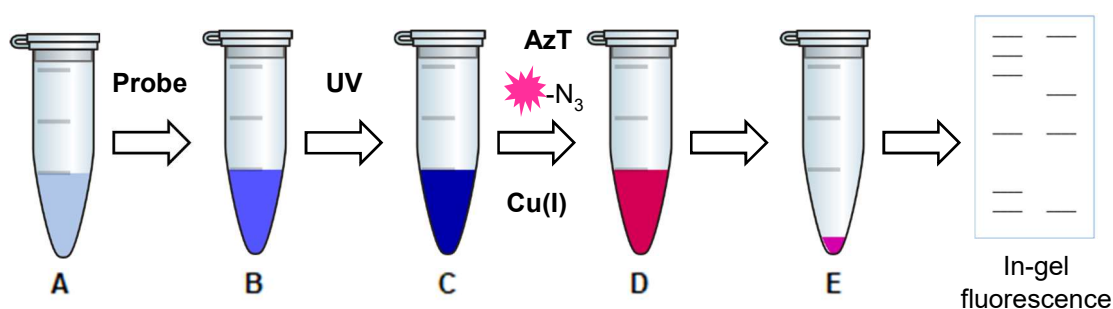


Figure 28: Work-flow for *Plasmodium falciparum* lysate labelling with photo-crosslinking probes and AzT for visualisation by in-gel fluorescence: A) *Pf*3D7 lysate is incubated with the probe; B) UV irradiation binds the probe to the target proteins in the lysate; C) AzT (Azide-TAMRA) is added under copper catalysed click-chemistry conditions; D) proteins are precipitated and washed; E) The proteins are re-dissolved and separated by SDS-PAGE. In-gel fluorescence can be used to visualise the labelled proteins.

The lysate was incubated with the probe at 4 °C to ensure the proteins remain folded and biologically active. UV irradiation at around 365 nm activates the diazirine and benzophenone functionalities (Table 3) and cross-links the probe to the proteins. The fluorescent azide-containing capture reagent AzT (Figure 29) was then conjugated by copper-catalysed click chemistry to the alkyne handles on the probe using copper sulfate, tris(2-carboxyethyl)phosphine (TCEP) and tris((1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl)amine (TBTA), as previously described.¹⁸⁶⁻¹⁸⁸ Finally, after protein precipitation, the proteins were dissolved in an appropriate buffer, separated by SDS-PAGE (which separates the proteins by molecular weight), and analysed from in-gel fluorescence. Full experimental details of the labelling procedure are given in the experimental section.

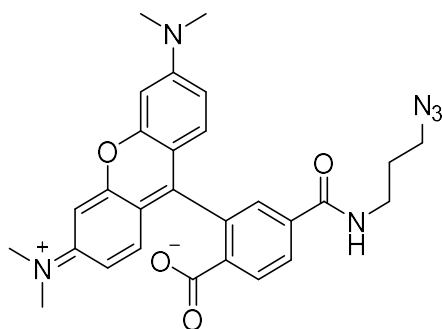


Figure 29: Fluorescent capture reagent TAMRA-Azide (AzT).

3.2.1.1. Initial Labelling

Initial experiments found that a lysate concentration of 3 mg mL⁻¹ and a probe concentration of 10 μM showed labelling of multiple protein bands with probes **2** and **4**, as shown in Figure 30, demonstrating the effectiveness of the probes to label proteins in the lysate.

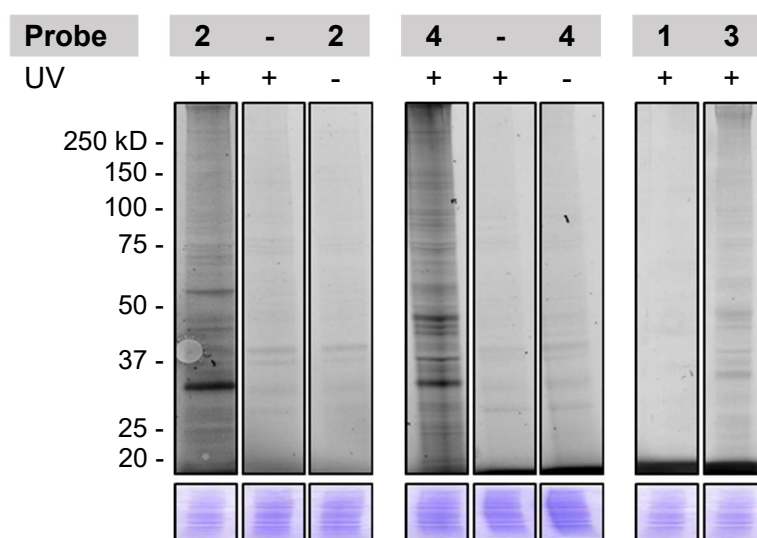


Figure 30: Initial labelling for probes **1-4** show probes **2** and **4** labelling a number of proteins in the lysate, with no labelling in the controls. *Pf*3D7 lysate was incubated with 10 μ M probe and the samples subjected to photo-crosslinking with UV (365 nm) and copper-catalysed click-chemistry with AzT. Fluorescence is shown in greyscale. All lanes contained 30 μ g of total protein and equal loading was confirmed by Coomassie brilliant blue staining (image in blue). Numbers in the left side indicate the molecular weights (in kDa) of proteins.

The DMSO controls showed no labelling, and an additional control experiment with no UV irradiation for photo-crosslinking also showed no labelling, suggesting both the photo-crosslinking and click chemistry of the fluorophore were working effectively.

Probe **1** showed no evidence of labelling, which may be due to limited binding given its low anti-*Plasmodium* activity (IC_{50} = 395 nM). Probe **3** showed some evidence of labelling, but probes **2** and **4** gave more robust and reliable labelling and were chosen for further experiments. These two probes were the most active in the cytotoxicity assay (Table 6), but other factors such as the photo-crosslinking efficiency and access to the alkyne handle for click chemistry after binding may also affect labelling efficiency. The protocols and labelling experiments related to probe **4** have recently been published.¹⁸⁴

3.2.2. Protocol Optimisation

3.2.2.1. Probe Concentration

In order to test the probes and optimise the labelling procedures, the concentration-dependence of labelling with probe concentrations of 0-100 μM was tested, as shown in Figure 31. Both probes show dose-dependent labelling.

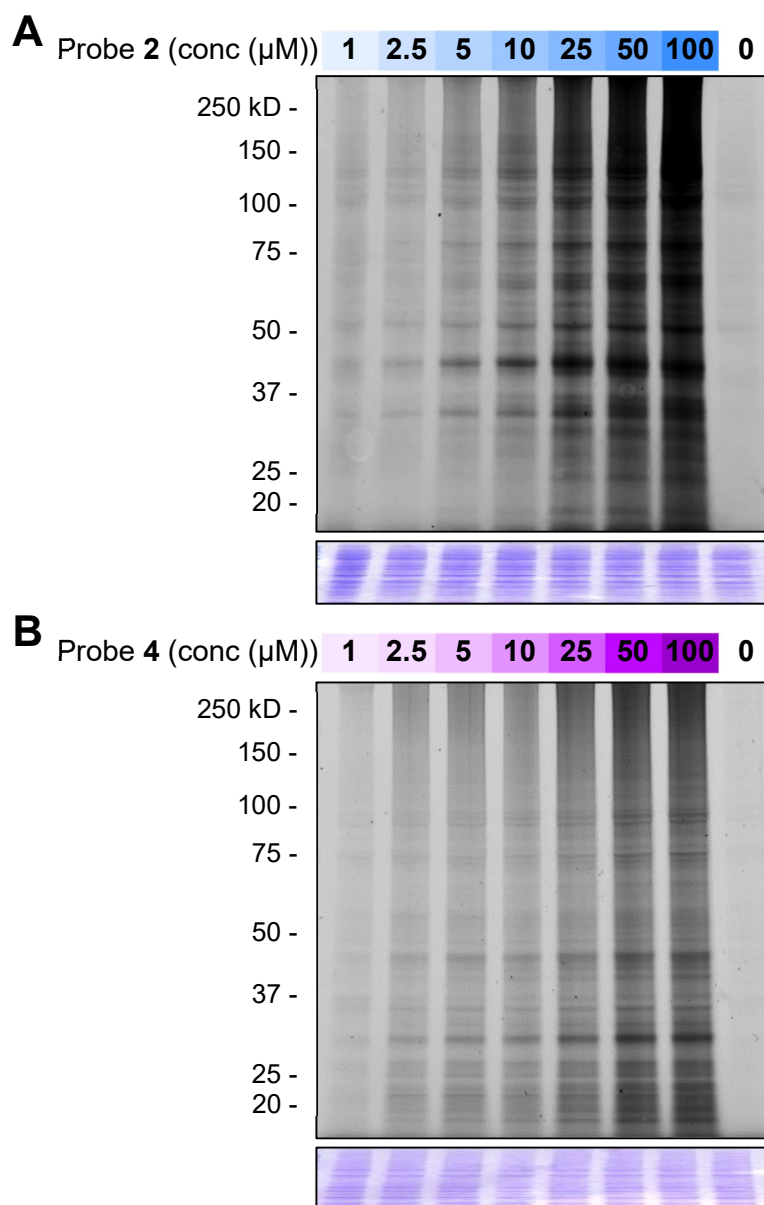


Figure 31: Dose-dependent labelling of *Plasmodium falciparum* lysate by (A) probe 2 and (B) probe 4 (published data¹⁸⁴). *Pf*3D7 lysate was incubated with 0-100 μM probe and the samples subjected to photo-crosslinking with UV (365 nm) and copper-catalysed click-chemistry with AzT. Fluorescence is shown in greyscale. All lanes contained 30 μg of total protein and equal loading was confirmed by Coomassie brilliant blue staining (image in blue). Numbers in the left side indicate the molecular weights (in kDa) of proteins.

From these results, a concentration of 10 μM was found to be optimal for labelling with probe **2** whilst 25-50 μM was found to be optimal for probe **4**, to obtain robust labelling of multiple bands without saturation. Whilst probe **4** has a higher cytotoxicity compared to probe **2**, when alkyl diazirines form the activated carbene intermediate, internal rearrangement and solvent insertion are common,¹⁸⁹ which may explain the need for a higher concentration of probe. Benzophenone intermediates also have a lower affinity for water.¹⁹⁰

3.2.2.2. Irradiation Time

To further optimise procedures, the effect of different irradiation times on labelling with both probes **2** and **4** were tested, as illustrated in Figure 32. With both probes, 20 minutes of irradiation was sufficient for labelling saturation. Hence the protocol was adapted to irradiate for 20 minutes, as prolonged irradiation can increase non-specific background labelling.

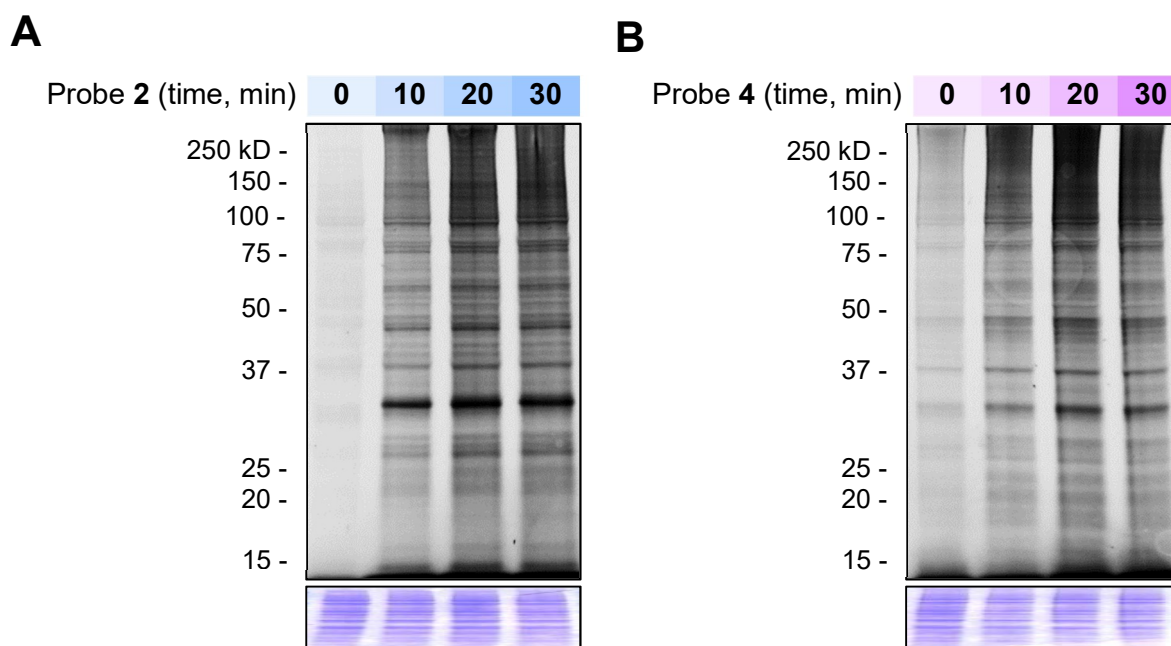


Figure 32: Irradiation time-dependence labelling of *Plasmodium falciparum* lysate by (A) probe **2** and (B) probe **4**. *Pf*3D7 lysate was incubated with 10 μM of probe **2** or 25 μM of probe **4** and the samples subjected to photo-crosslinking with UV (365 nm) and copper-catalysed click-chemistry with AzT. Fluorescence is shown in greyscale. All lanes contained 30 μg of total protein and equal loading was confirmed by Coomassie brilliant blue staining (image in blue). Numbers in the left side indicate the molecular weights (in kDa) of proteins.

3.2.3. Competition Experiments

The optimised protocol and initial results demonstrated the effectiveness of the probes to label a variety of proteins within the lysate. However, given the irreversible nature of photo-crosslinking, some of these labelled bands may be non-specific targets. To begin to establish specific targets, and to establish if the probes are binding to the same targets as the diaminoquinazoline series, competition experiments using representative compounds from the series were carried out, to see if active compounds could out-compete the probe at increasing concentrations.

3.2.3.1. Competition Experiments with BIX-01294

Initially, BIX-01294 (IC_{50} = 43 nM) was chosen, since it is a potent compound from the series, and the most analysed to date. It was used at increasing concentrations in the labelling experiments, with both probe **2** and probe **4**. For specific BIX-01294 targets, a reduction in labelling would be expected, as the inhibitor out-competes the probe. Due to the reversible binding of the inhibitors compared to the irreversible nature of the probe photo-crosslinking, high concentrations of BIX-01294 are needed to observe these effects.

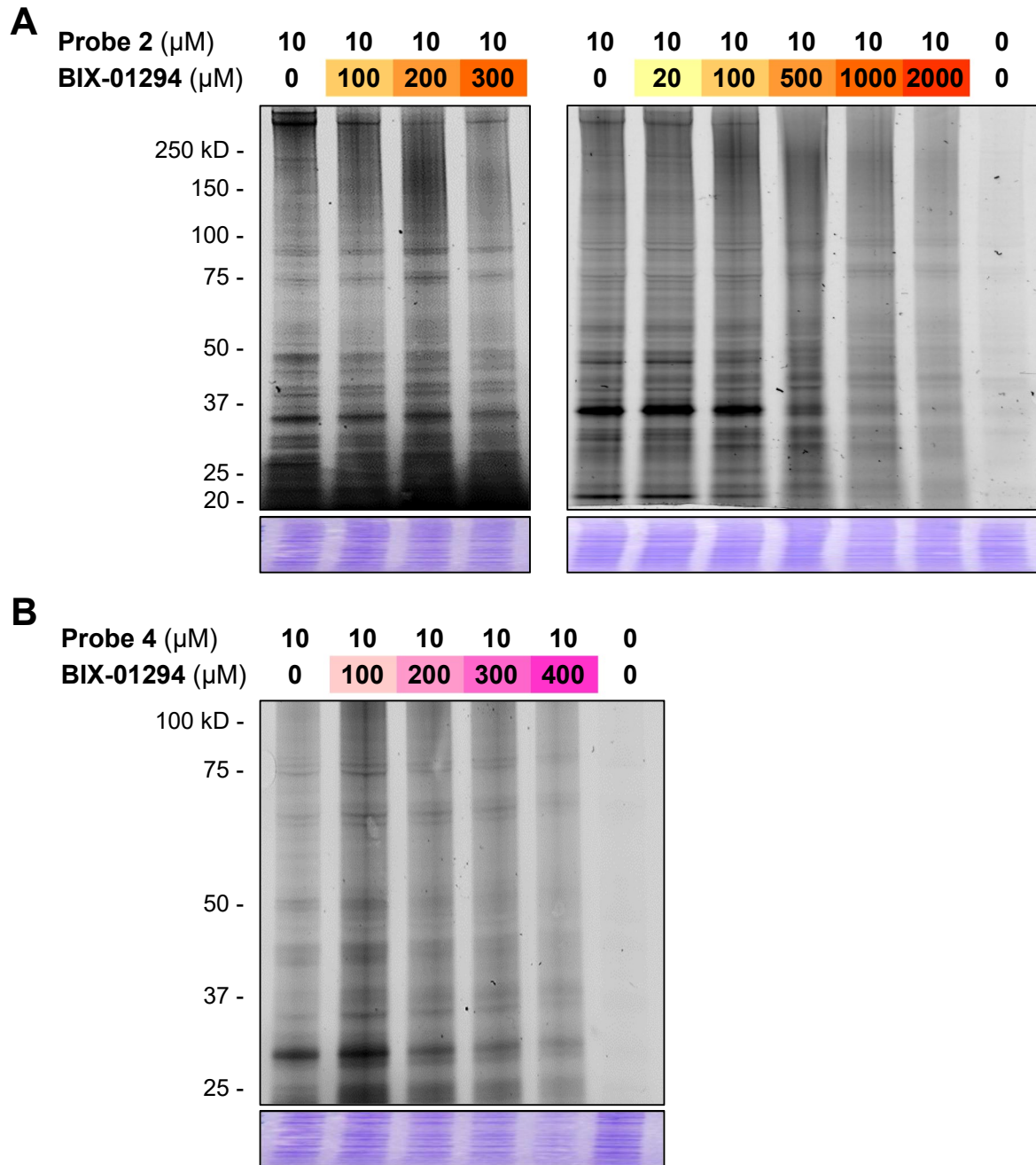


Figure 33: Competition of (A) Probe 2 and (B) Probe 4 (published data¹⁸⁴) with increasing concentrations of BIX-01294 (0-2000 μM) in *Plasmodium falciparum* lysates. *Pf*3D7 lysate was incubated with BIX-01294 followed by 10 μM of the probe and the samples subjected to photo-crosslinking with UV (365 nm) and copper-catalysed click-chemistry with AzT. Fluorescence is shown in greyscale. All lanes contained 30 μg of total protein and equal loading was confirmed by Coomassie brilliant blue staining (image in blue). Numbers in the left side indicate the molecular weights (in kDa) of proteins

BIX-01294 (0-2000 μ M) was incubated with the *Plasmodium falciparum* lysate for 30 minutes before the addition of the probe. As depicted in Figure 33 there is a general attenuation in fluorescence of labelled bands with increasing concentrations of BIX-01294.

These results suggest that both probe **2** and probe **4** have similar proteome target profiles to BIX-01294, as anticipated from the SAR, and that both probes could be useful tools for the identification of the targets of this compound series.

3.2.3.2. Competition Experiments with Other Inhibitors

Following on from these experiments, a similar competition experiment was carried out using other representative compounds from the diaminoquinazoline series, with a range of potencies, as shown in Figure 34.

Several potent compounds from the library were chosen (green), alongside two negative controls (red) that have low potency against *Plasmodium falciparum*. The potent compounds were able to out-compete probe **2**, leading to a reduction in fluorescence of a number of bands, whereas the inactive compounds were not, as would be expected if the probe has a similar proteome target profile to the compound series.

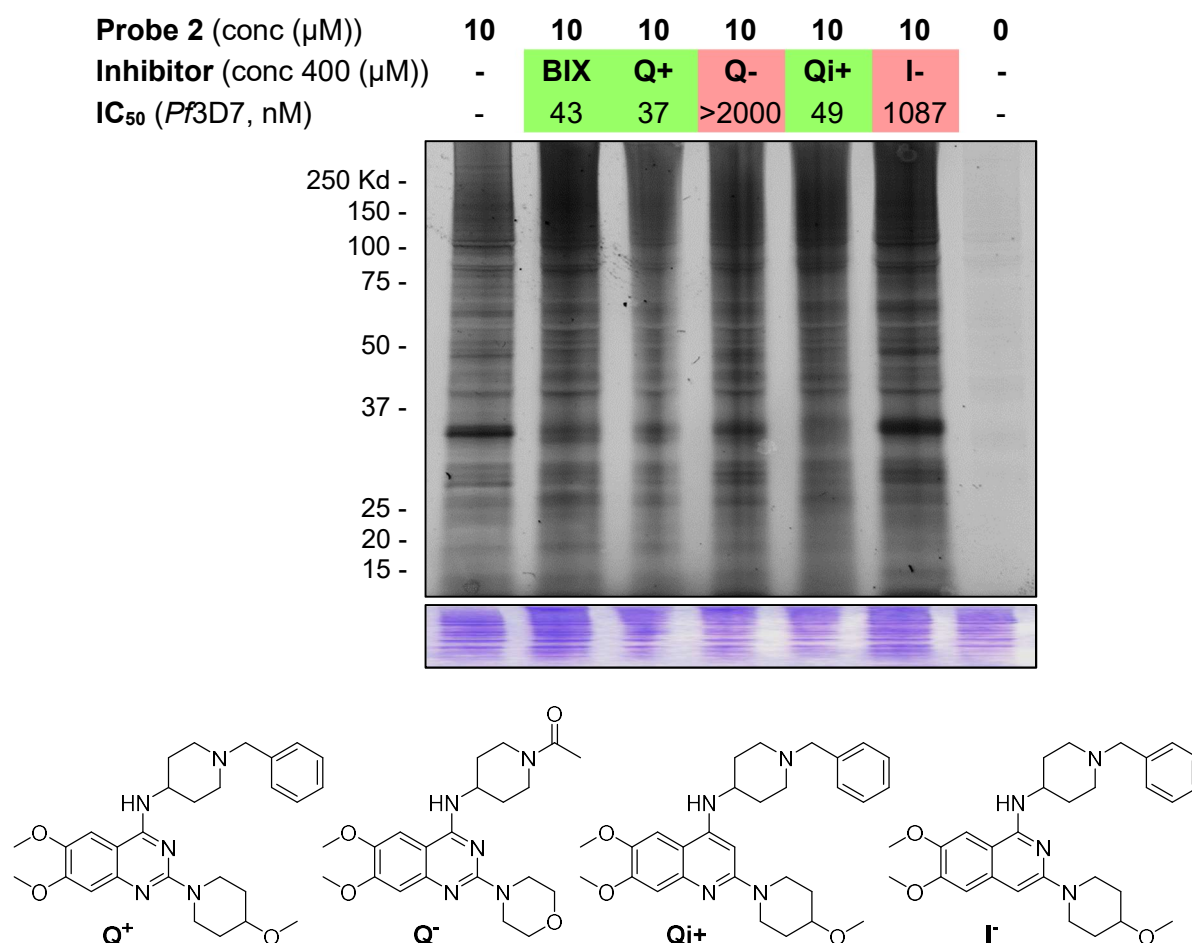


Figure 34: Competition of Probe 2 by a number of representative inhibitors of varying potencies in *Plasmodium falciparum* lysates. Pf3D7 lysate was incubated with the competitive compound (400 μM) followed by 10 μM of probe 2 and the samples subjected to photo-crosslinking with UV (365 nm) and copper-catalysed click-chemistry with AzT. Fluorescence is shown in greyscale. All lanes contained 30 μg of total protein and equal loading was confirmed by Coomassie brilliant blue staining (image in blue). Numbers in the left side indicate the molecular weights (in kDa) of proteins.

The results from the in-gel fluorescence experiments show that a number of proteins can be labelled with the probes in *Plasmodium falciparum*, and that the probes 2 and 4 appear to have similar proteome target profiles to the diaminoquinazoline compound series of interest, suggesting these are suitable tools for target identification.

3.3. Proteomics

In order to identify the targets of these series, the probes were used to pull-down proteins which were then analysed using mass-spectrometry for protein identification, using a shotgun proteomics approach.¹⁴⁸⁻¹⁵²

3.3.1. Protocol Development

In order to identify the targets engaged by the probes, the workflow was altered, replacing the AzT tag with azide-TAMRA-Biotin (AzTB, Figure 35).¹⁸⁷⁻¹⁸⁸ This versatile reagent contains the same TAMRA fluorophore for protein visualization as AzT, but also a biotin subunit to allow for enrichment of the labelled proteins by NeutrAvidin 'pull-down', due to the strong interaction between avidin and biotin.¹⁹¹ NeutrAvidin is deglycosylated avidin, that reduces non-specific binding compared with avidin.¹⁹²

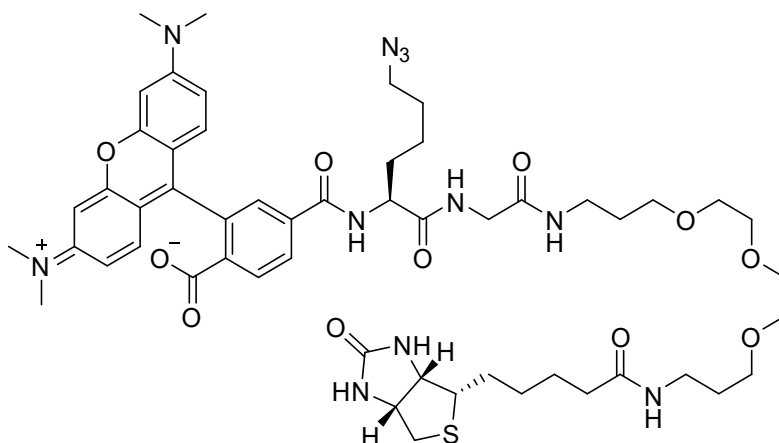


Figure 35: Structure of azide-TAMRA-Biotin (AzTB),¹⁸⁷⁻¹⁸⁸ a capture reagent containing both a fluorophore for visualisation, and a biotin for pull-down.

The lysate was incubated with the probes (or DMSO as a control), photo-crosslinked and conjugated to AzTB with copper catalysed click chemistry, before incubation with NeutrAvidin beads and stringent washing.¹⁸⁷ Proteins were released from the beads by

boiling in SDS loading buffer. In order to assess that the pull-down was working, probe-labelled proteins were analysed by in-gel fluorescence before and after pull-down.

As shown by Figure 36, the fluorescence is maintained after pull-down, with very little fluorescence in the supernatant obtained after incubation with the beads. Staining with coomassie shows that the majority of proteins from the lysate were not pulled-down.

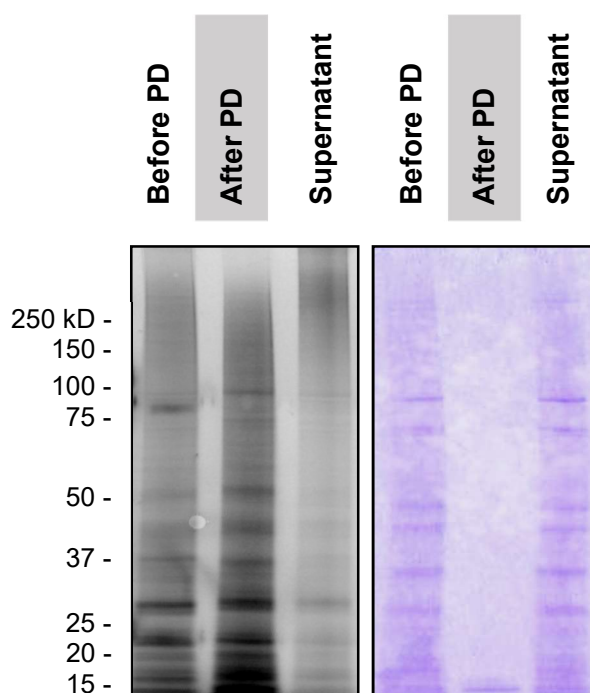


Figure 36: In-gel fluorescence before and after pull-down (PD) with probe **2** and NeutrAvidin-agarose beads. *Pf3D7* lysate was incubated with probe **2** (10 μ M) and the samples subjected to photo-crosslinking with UV (365 nm) and copper-catalysed click-chemistry with AzTB. Pull-down was done with NeutrAvidin-agarose beads. Fluorescence is shown in greyscale and protein loading with Coomassie brilliant blue staining (image in blue). Numbers in the left side indicate the molecular weights (in kDa) of proteins.

This is an important result, as it shows that the pull-down is working, and that it is the proteins labelled with the probe and hence AzTB that are being pulled down by the beads, whilst the majority of proteins are not labelled. Hence, this protocol enriches the sample for the apparent target proteins of these probes and the diaminoquinazoline series, and so could be used for proteomic analysis. For this, the captured proteins were instead subjected

to on-bead reduction and alkylation to aid unfolding and cleavage, and then trypsin digestion before the obtained peptides were analysed by mass spectrometry.^{187,193-194}

3.3.2. Testing the Protocol

To test the protocol, three pull-down experiments were prepared without replicates: one with probe **2** (10 μ M), one with probe **4** (25 μ M) and a DMSO control. Lysate (600 μ g protein) was incubated with the probes, conjugated to AzTB, and finally incubated with NeutrAvidin-agarose beads using the protocols developed above. The beads were washed stringently, with SDS in phosphate buffered saline (PBS), urea in PBS, and ammonium bicarbonate. This was followed by reduction with dithiothreitol (DTT), alkylation with iodoacetamide and overnight trypsin digestion. The peptides were then desalted,¹⁹⁵ and prepared for mass spectrometry analysis.

For this initial analysis, a low-resolution mass spectrometer (LTQ Velos Pro linear ion trap LC-MS system) was used to test the final probe of the protocol and therefore our ability to identify proteins. Although the use of this mass spectrometer together with the fact that no replicates were analysed, means this initial data is not sufficient to claim statistical significance of the results, it allowed some initial analysis.

Around 150-250 proteins were identified in each of the three samples, with some common proteins between all three. Table 7 shows the top ten proteins identified by probe **2** but not in the control sample. Two of these proteins were also identified by probe **4**. Promisingly, one of the HKMTs, *PfSET1* was identified by probe **2**, although significant further evidence would be needed to validate this result.

Table 7: The top 10 proteins identified by pull-down using AzTB by probe **2** but not present in the control sample. Two of these proteins (grey) were also identified by probe **4**. *Pf*SET1 was identified by probe **2** (green).

	Protein Description
1	Enolase (PF3D7_1015900)
2	Heat shock protein 90 (PF3D7_0708400)
3	Pyruvate kinase (PF3D7_0626800)
4	E3 ubiquitin-protein ligase, putative (PF3D7_0826100)
5	Ornithine aminotransferase (PF3D7_0608800)
6	Putative histone-lysine N-methyltransferase SET1 (PF3D7_0629700)
7	Importin-7, putative (PF3D7_0706000)
8	Uncharacterised protein (PF3D7_1218500)
9	14-3-3 protein (PF3D7_0818200)
10	Uncharacterised protein (PF3D7_0704000)

This preliminary data shows again that the pull-down is working and suitable for proteomics. Moving forward, experiments were carried out in triplicate, and analysed using a nano LC-MS/MS (liquid chromatography tandem mass spectrometry) on a high resolution Orbitrap mass spectrometer,^{187,193-194} to provide high resolution data.

3.3.3. Proteomics Data: Probes 2 and 4 in Lysate

To this end, samples were prepared as stated above using either probe **2** (10 μ M), probe **4** (50 μ M) or DMSO as a control, and the resulting peptides were analysed by high resolution tandem mass spectrometry.^{187,193-194} All experiments were carried out in triplicate to minimise false positives and to obtain statistically significant results.

The mass spectrometry data was then processed and analysed using MaxQuant, specialist software that incorporates a set of algorithms specifically designed for peptide and protein identification. MaxQuant has its own search engine incorporated, Andromeda, to identify the

obtained peptides after comparing them with the *P. falciparum* genome thanks to their fragmentation pattern.¹⁹⁶⁻¹⁹⁸ This processing provided us with label-free quantification (LFQ) data,¹⁹⁷ that was analysed using Perseus, a platform developed for this specific purpose.¹⁹⁹ Further details can be found in the experimental section. The data and analysis from probe **4** has recently been published.¹⁸⁴

After processing the data, the first thing to take into consideration was the correlation between the LFQ intensities of the replicates for each experiment (Figure 37). As shown in Figure 37A, there was a good correlation between the triplicates with probe **2** and the triplicates with the DMSO control, which gives confidence to continue with the analysis. However, for the experiment with probe **4**, one of the replicates in the probe treated samples, (Probe **4** - 2), did not show good correlation with the others (Figure 37B), and hence this replicate was eliminated for further analysis.

Figure 38 shows the same results but represented using heatmaps instead. These plots show the identified proteins for each of the samples colour-coded based on their LFQ intensity from blue (low intensity) to red (high intensity). Therefore, when there is good correlation between replicates a similar colour distribution is observed, as shown in Figure 38A. However, Figure 38B shows how one of the replicates in the probe **4** treated samples, and one of the DMSO controls don't correlate properly with the other two, presenting more low intensity proteins (blue). This probably means that there was a problem in the preparation of these two samples and as a result fewer amounts of proteins in general have been obtained. It is important to highlight that although some of the proteins have similar intensity in both the probe samples and the DMSO controls, and therefore these are most likely unspecific proteins, most of the detected peptides are found with much higher intensity in the probe samples when compared to the DMSO controls (yellow or orange vs blue). These proteins are enriched by the probes, and hence may be targets of the diaminoquinazoline compound series.

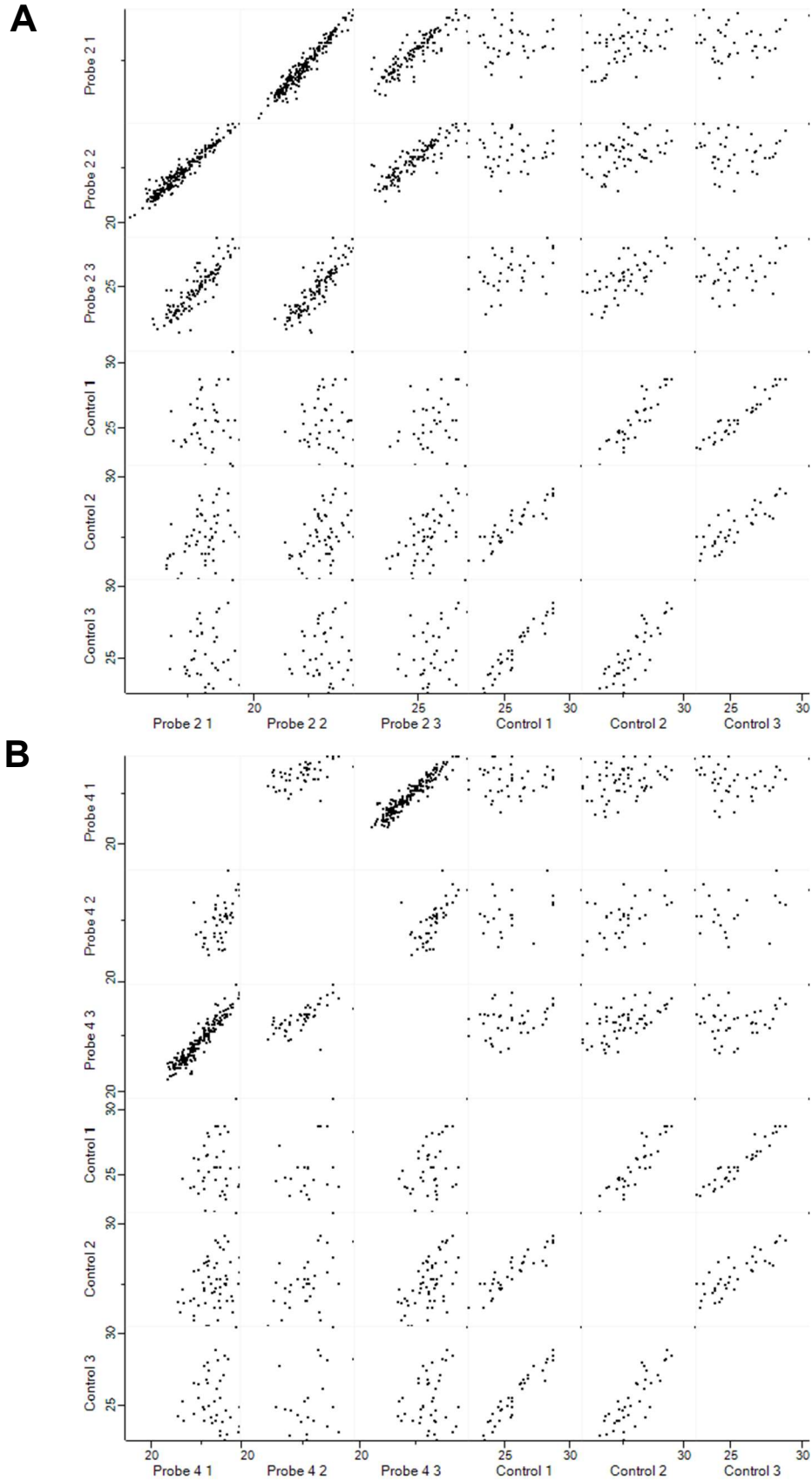


Figure 37: Correlation diagrams of the proteins identified by pull-down experiments with AzTB and NeutrAvidin: (A) triplicates from pull-down with probe **2** (B) triplicates from pull-down with probe **4** showing the second experiment as an anomaly. Controls were carried out with DMSO.

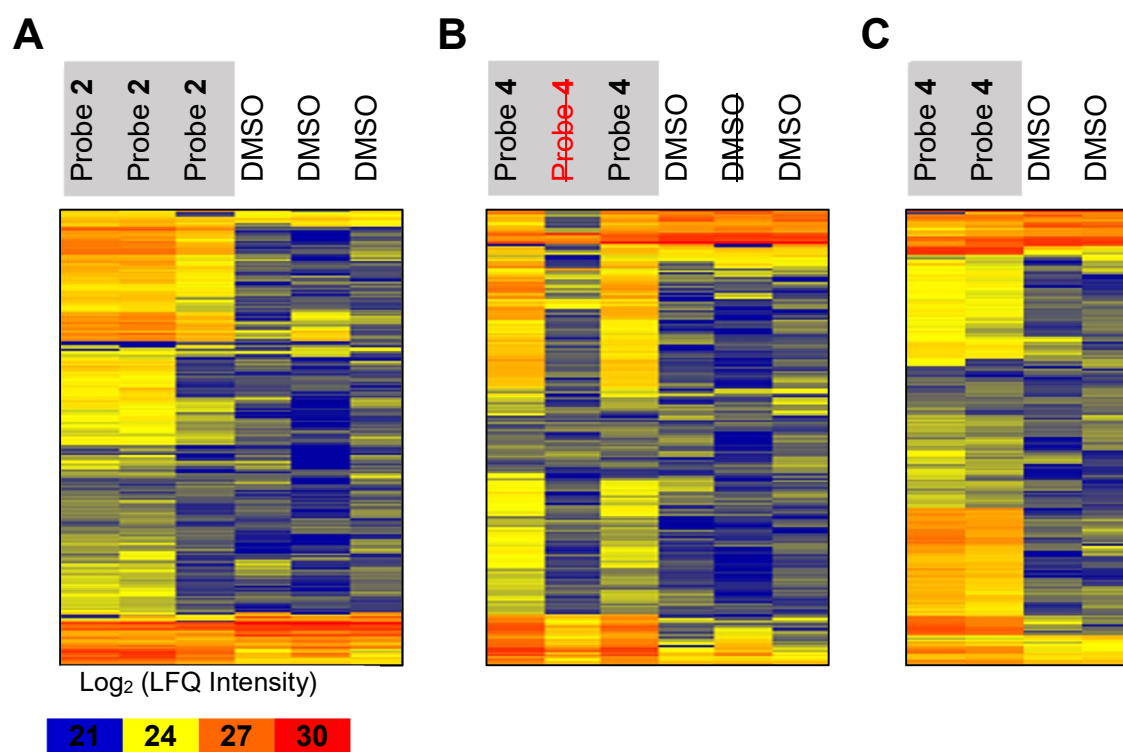


Figure 38: Heatmap for all proteins found in terms of their relative abundance $\log_2(\text{LFQ intensity})$ from Euclidian hierarchical row clustering with Perseus 1.5.6.0. High intensity is shown in red and low intensity in blue. (A) Heat map for the triplicates performed with probe 2; (B) Heat map for the triplicates performed with probe 4, including the anomalous repeat; (C) Heat map for the duplicates performed with probe 4 excluding the anomalous result.

After analysing the success of the experiment by looking at the correlation between replicates, the next step was to identify which of the detected proteins are significantly enriched by the probes compared to the DMSO control. Thus, a two-sample student's t-test was carried out for each probe. In total, 57 and 104 proteins were found to be significantly enriched by probes 2 and 4 respectively (Figure 39 and Figure 40). Comparing the LFQ intensities for these enriched proteins in the probe samples compared to the same proteins in the controls, confirms that the intensities of these proteins were significantly higher in the probe samples (Figure 41).

To assess the importance of these significantly enriched proteins, they were analysed for essentiality using the PhenoPlasm database (<http://phenoplasm.org/>).²⁰⁰ A protein is considered to be essential if the gene encoding it cannot be successfully disrupted without

the parasite dying as a consequence. Therefore, this analysis should provide us with some information about the importance of the identified proteins for *P. falciparum* survival.

Of the 57 proteins enriched by probe **2**, and the 104 enriched by probe **4** there is only information regarding the genetic disruption of 6 and 5 of them, respectively, with three of them being essential in each case (filled purple diamonds vs unfilled purple diamonds in Figure 39 and Figure 40, respectively).

Although the proteomic experiments were carried out using *P. falciparum* lysates, the PhenoPlasm database provides information of all the studies available for a given gene ID across the *Plasmodium* spp.²⁰⁰ The genes with no essentiality information from *P. falciparum* were analysed for phenotypic information from *P. berghei* homologues (a rodent strain), since a much more thorough functional profiling of *P. berghei* has been carried out compared to *P. falciparum* (2700 genes versus 398). Thus, information for 27 and 53 additional genes was found with probes **2** and **4**, respectively (Figure 39 and Figure 40, green). Of them, 22 and 39 were found to be essential, respectively (filled diamonds vs unfilled diamonds for non-essential proteins). For the remaining proteins, no known essentiality information was available (Figure 39 and Figure 40, blue).

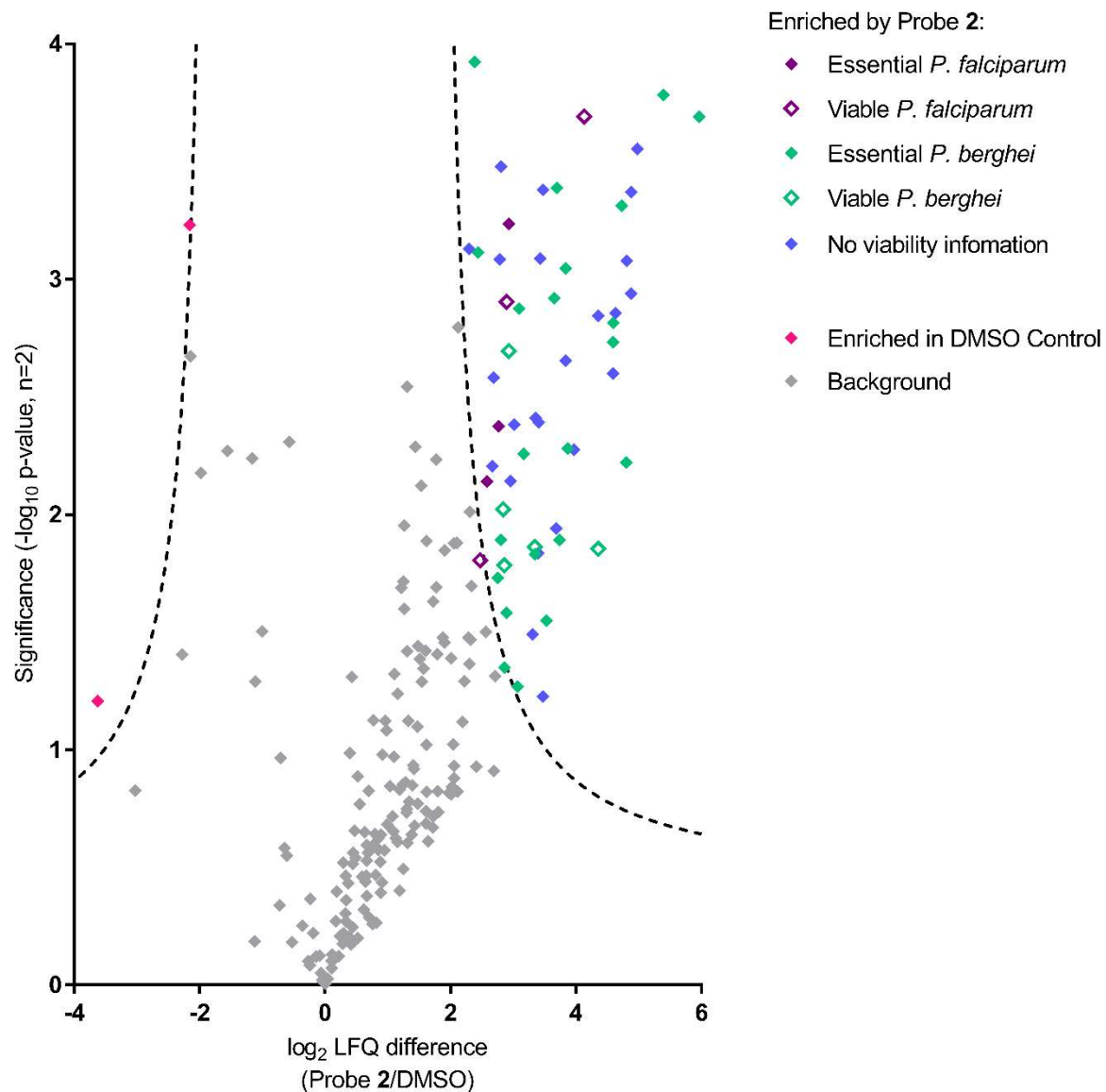


Figure 39: Volcano plot from analysis with Perseus 1.5.6.0 using t-test significance testing of triplicate results with FDR = 0.01 (False Discovery Rate) and $S_0 = 2$. 57 proteins were enriched in the samples containing probe 2 (right-hand side: blue, purple and green) compared to the 2 significantly relevant proteins obtained in the DMSO controls (left-hand side: pink).

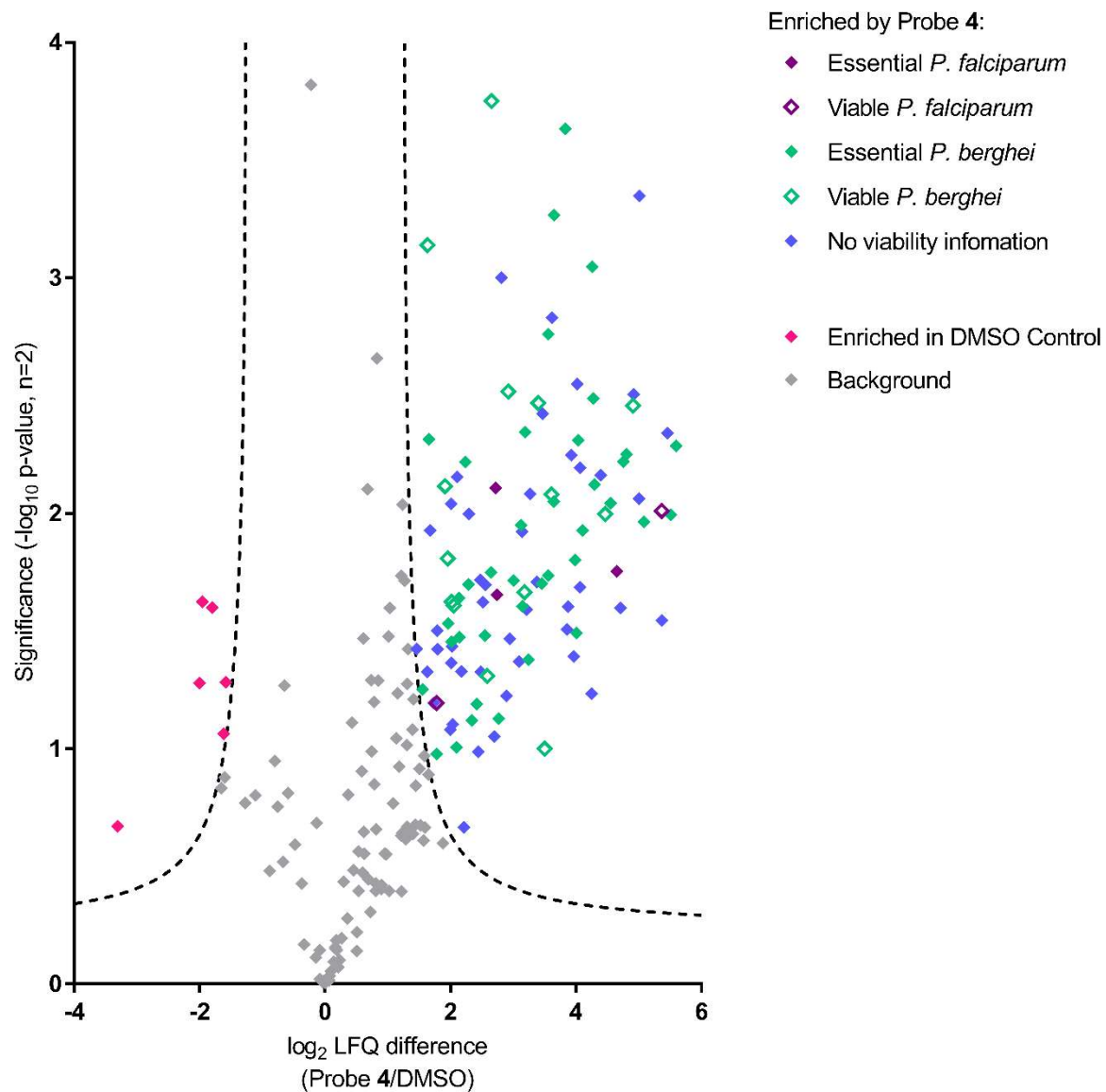


Figure 40: Volcano plot from analysis with Perseus 1.5.6.0 using t-test significance testing of duplicate results with FDR = 0.001 (False Discovery Rate) and $S_0 = 2$. 104 proteins were enriched in the samples containing probe 4 (right-hand side: blue, purple and green) compared to the 6 significantly relevant proteins obtained in the DMSO controls (left-hand side: pink). (published data¹⁸⁴)

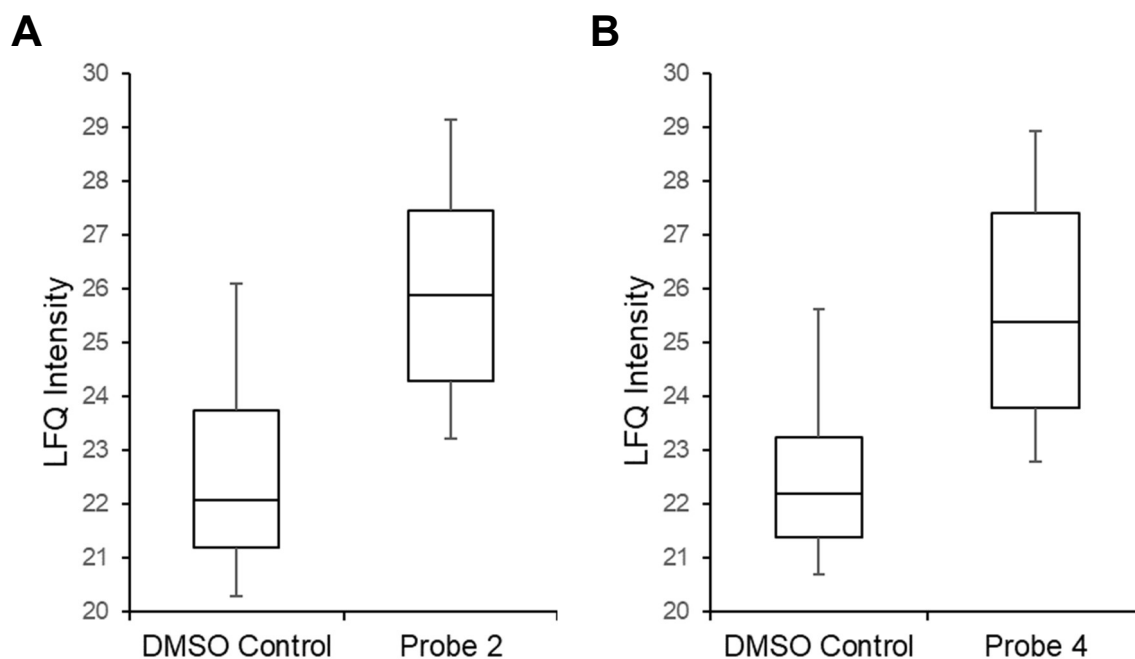


Figure 41: Box plots of (A) the 57 proteins significantly enriched for probe **2** and (B) the 104 proteins significantly enriched for probe **4**. The plots show the spread of intensities of the identified proteins around the median, for the control samples (left) and the probe samples (right), with 80% of the data contained within the marked box. Intensities for the probes are significant compared to the background from the control.

3.3.3.1. Data Analysis: Comparing Probes 2 and 4

There were 57 proteins enriched by probe **2** compared with 104 enriched by probe **4**. However, since both these probes maintain antimalarial activity, with an IC_{50} of 128 nM and 42 nM respectively, it is reasonable that the proteins enriched by both probes are more representative of the proteins targeted by the diaminoquinazoline series.

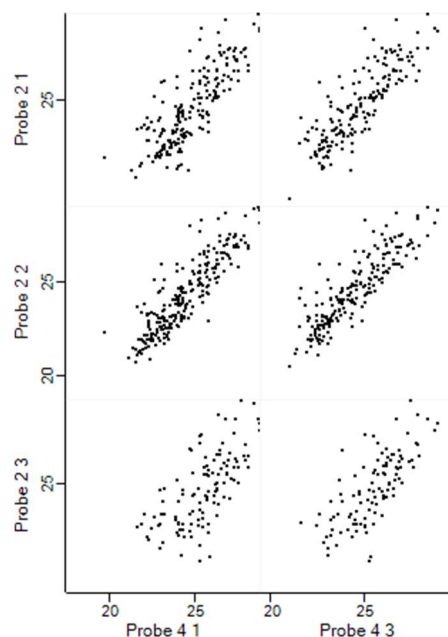


Figure 42: Correlation diagrams of the proteins identified by pull-down experiments with AzTB and NeutrAvidin by probe **2** and probe **4**.

Figure 42 shows the correlation between the proteins enriched by probe **2** and probe **4**. There is reasonable correlation between the two probes, suggesting that they have similar target profiles, as anticipated from the SAR of this series. The fact that these probes appear to behave as the SAR predicts is promising for the use of these probes to identify targets of the diaminoquinazoline series, as combined with the earlier competition experiments, the data suggests they have a representative target profile.

Table 8 shows the 45 proteins that were identified as significantly enriched by both probe **2** and probe **4** compared to the DMSO control, along with the known essentiality information.

Table 8: The 45 proteins significantly enriched after AzTB and NeutrAvidin pull-down by both probe **2** and probe **4** compared to the DMSO control.

Gene	Description	Essentiality <i>P. falciparum</i> <i>P. berghei</i>
PF3D7_0708800	heat shock protein 110	Y
PF3D7_1451100	elongation factor 2	Y
PF3D7_1447000	40S ribosomal protein S5	Y
PF3D7_1342000	40S ribosomal protein S6	Y
PF3D7_1338300	elongation factor 1-gamma, putative	Y
PF3D7_1358800	40S ribosomal protein S15	Y
PF3D7_1246200	actin I	Y
PF3D7_1324900	L-lactate dehydrogenase	Y
PF3D7_1224300	polyadenylate-binding protein, putative	Y
PF3D7_1311800	M1-family alanyl aminopeptidase	Y
PF3D7_1331800	60S ribosomal protein L23, putative	Y
PF3D7_1011800	PRE-binding protein	Y
PF3D7_1015900	Enolase	Y
PF3D7_1008700	tubulin beta chain	Y
PF3D7_1004000	60S ribosomal protein L13, putative	Y
PF3D7_0922500	Phosphoglycerate kinase	Y
PF3D7_0813900	40S ribosomal protein S16, putative	Y
PF3D7_0827900	Protein disulfide-isomerase	Y
PF3D7_0617200	conserved protein, unknown function	Y
PF3D7_0626800	pyruvate kinase	Y
PF3D7_0818200	14-3-3 protein	Y
PF3D7_1343000	phosphoethanolamine N-methyltransferase	N
PF3D7_0608800	ornithine aminotransferase	N
PF3D7_1346300	DNA/RNA-binding protein Alba 2	N
PF3D7_1347500	DNA/RNA-binding protein Alba 4	N
PF3D7_0708400	heat shock protein 90	N
PF3D7_0524000	karyopherin beta	N
PF3D7_0513300	purine nucleoside phosphorylase	N
PF3D7_1468700	eukaryotic initiation factor 4A	?
PF3D7_1465900	40S ribosomal protein S3	?

PF3D7_1424400	60S ribosomal protein L7-3, putative	?
PF3D7_1414300	60S ribosomal protein L10, putative	?
PF3D7_1424100	60S ribosomal protein L5, putative	?
PF3D7_1357000	Elongation factor 1-alpha	?
PF3D7_1105400	40S ribosomal protein S4, putative	?
PF3D7_1027800	60S ribosomal protein L3	?
PF3D7_0922200	S-adenosylmethionine synthase	?
PF3D7_0813300	conserved protein, unknown function	?
PF3D7_0516900	60S ribosomal protein L2	?
PF3D7_0322900	40S ribosomal protein S3a	?
PF3D7_1006800	single-strand telomeric DNA-binding protein GBP2, putative	?
PF3D7_1012400	hypoxanthine-guanine phosphoribosyltransferase	?
PF3D7_1309100	60S ribosomal protein L24, putative	?
PF3D7_0317600	40S ribosomal protein S11, putative	?
PF3D7_0500800	mature parasite-infected erythrocyte surface antigen	?

Only one of these proteins is known to be essential in *P. falciparum*: heat shock protein 110, which prevents aggregation of asparagine-rich proteins, and allows the parasite to survive the high temperatures and stressful conditions associated with malarial infection.²⁰¹

Another protein enriched by both probes is *P. falciparum* phosphoethanolamine *N*-methyltransferase (*PPMT*), an enzyme which uses SAM as a substrate to methylate ethanolamine phosphate and is involved in phospholipid synthesis in the parasite.²⁰² Whilst knock-out of this enzyme is viable, this has severe implications for parasite development and survival.²⁰³ This protein is important for gametocyte development and transmission,²⁰⁴ and has no human homologues,²⁰² making it an interesting potential target for drug discovery.

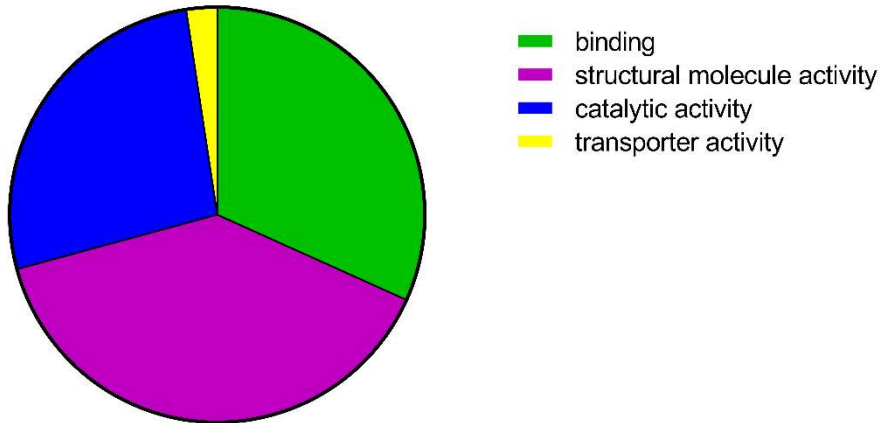
A number of proteins were enriched by both probes and found to be essential in *P. berghei*, and could be interesting targets for drug discovery, if they are specific targets of these probes. PRE-binding protein (PREBP) is a transcription factor involved in gene activation in the erythrocytic lifecycle of the parasite.³² *P. falciparum* M1-family alanyl aminopeptidase

(PfA-M1) is an essential protein involved in haemoglobin digestion,²⁰⁵ and inhibitors can halt parasite growth.²⁰⁶ L-lactate dehydrogenase (LDH) is a vital enzyme in almost all cells, but differences in the structure of *Plasmodium* LDH compared with humans could make it a possible target for drug discovery.²⁰⁷

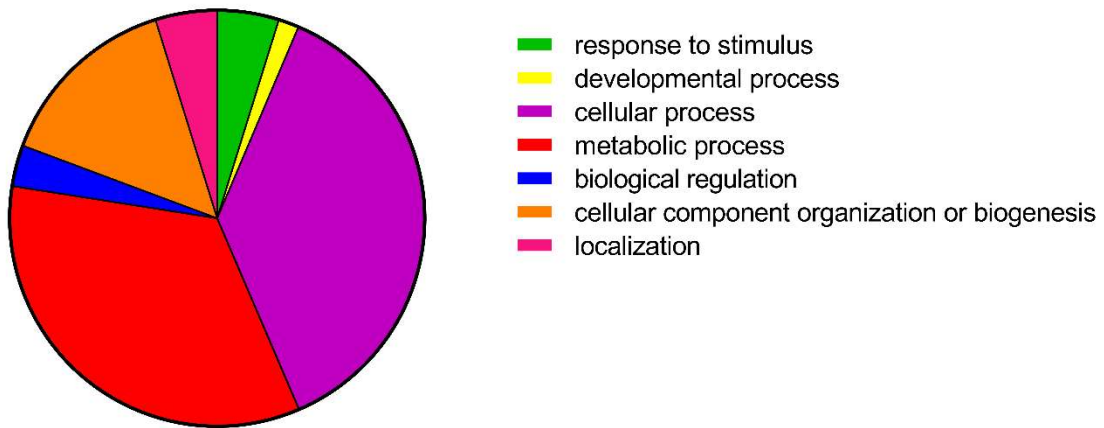
A number of the proteins in this list may be non-specific targets of these probes, possibly due to their high abundance in the lysate. Quantitative proteomics analysis of *P. falciparum* by Nirmalan *et al.* identified a number of these proteins as highly abundant, including LDH, PfPMT, 14-3-3 protein, actin-I and the putative kinase.²⁰⁸ Heat shock proteins, ribosomal proteins and elongation factors are also known to be highly abundant, and so are likely to be non-specific targets. Further validation of all potential targets will be required.

As well as looking at individual proteins enriched by both probes, these 45 proteins were analysed using the Protein ANalysis THrough Evolutionary Relationships (PANTHER) classification system.²⁰⁹⁻²¹¹ Figure 43A shows these proteins have a number of different molecular functions, the largest being binding, structural and catalytic activity. The binding category is large due to the number of ribosomal proteins identified. This may be due to a high abundance of ribosomal proteins present in the lysate used. Figure 43B shows that the enriched proteins are involved in a large number of biological processes in the parasite, in particular cellular and metabolic processes. The largest protein classes enriched (Figure 43C) were nucleic acid binding proteins and enzymes. The abundance of ribosome proteins in the schizont lysate may again account for the former, but the latter class could represent some interesting specific targets of these probes. In order to confirm the specific and non-specific targets of these probes, further evidence is needed.

A Molecular Function



B Biological Processes



C Protein Class

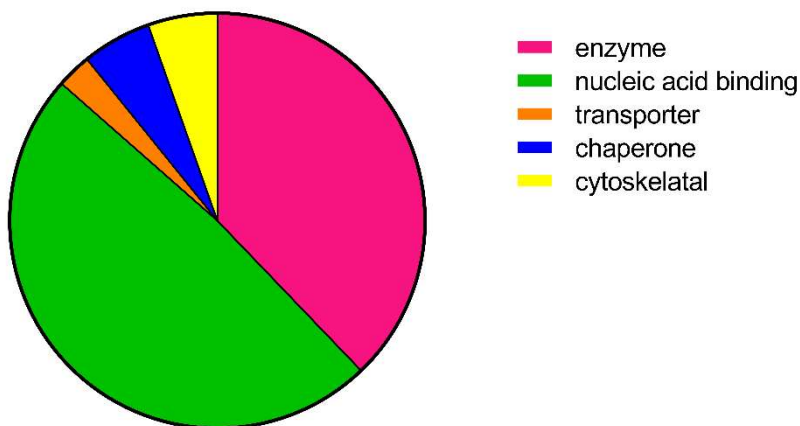


Figure 43: Analysis of the 45 significant targets engaged by both probe **2** and probe **4** based on gene ontology (GO) annotations using the protein analysis through the evolutionary relationships (PANTHER) classification system, which includes both experimental data and bioinformatics algorithms.²⁰⁹⁻²¹¹ Analysis of (A) molecular function; (B) biological processes and (C) protein class.

3.3.4. Proteomics Data: Probes 2 in Nuclear Extract

Notably, the *Pf*HKMTs are absent from this list, and indeed were not identified in any of the proteomic experiments, even as non-specific targets. One explanation for this is that the HKMTs are not the targets of the probes and inhibitor series. However, the *Pf*HKMTs are low abundance nuclear proteins, and so the absence of these proteins may be due to there not being sufficient quantities present in the lysate for detection. In an attempt to overcome this obstacle, a lysate fractionation was performed to separate out the nuclear and cytoplasmic proteins,²¹²⁻²¹³ before carrying out the pull-down proteomics experiment using probe **2**.

Figure 44 shows the comparison between labelling in the lysate before fractionation, the nuclear extract and the cytoplasmic extract, after incubation with probe **2**, photo-crosslinking, and click chemistry with AzT as described before. This experiment showed that the nuclear extract was in good condition to be used in pull-down proteomic experiments, and also enabled the adjustment of the experiment conditions. Because the nuclear extract is heavily enriched for the proteins it contains, it can be used at a much lower protein concentration (0.3 mg mL^{-1}).

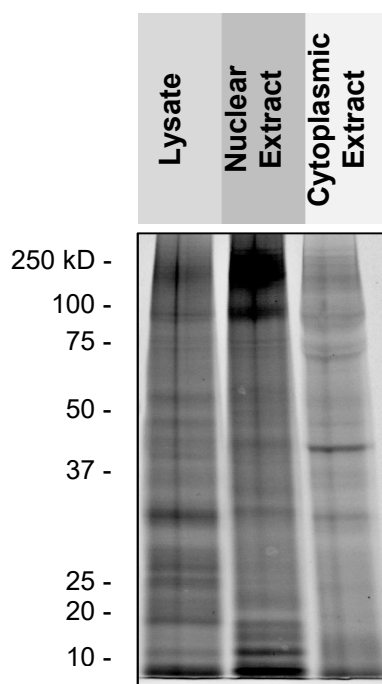


Figure 44: *Pf3D7* lysate, nuclear extract and cytoplasmic extract were incubated with 10 μ M of probe **2** and the samples subjected to photo-crosslinking with UV (365 nm) and copper-catalysed click-chemistry with AzT. Fluorescence is shown in greyscale. Numbers in the left side indicate the molecular weights (in kDa) of proteins.

Triplicates of nuclear extract (60 μ g protein) were treated with probe **2** (10 μ M) or DMSO as a control, and were crosslinked, conjugated to AzTB, incubated with NeutrAvidin beads and washed stringently as in previous pull-down experiments. This was followed by reduction, alkylation and digestion before mass spectrometry analysis. The obtained results were analysed as previously described using MaxQuant and Perseus.

As depicted in Figure 45A, there is reasonable correlation between the triplicates for the probe and control samples, albeit very few proteins were detected in the control samples. Indeed, the heatmap representation of the identified proteins shows that most of them are significantly enriched in the probe samples compared to the control ones (Figure 45B).

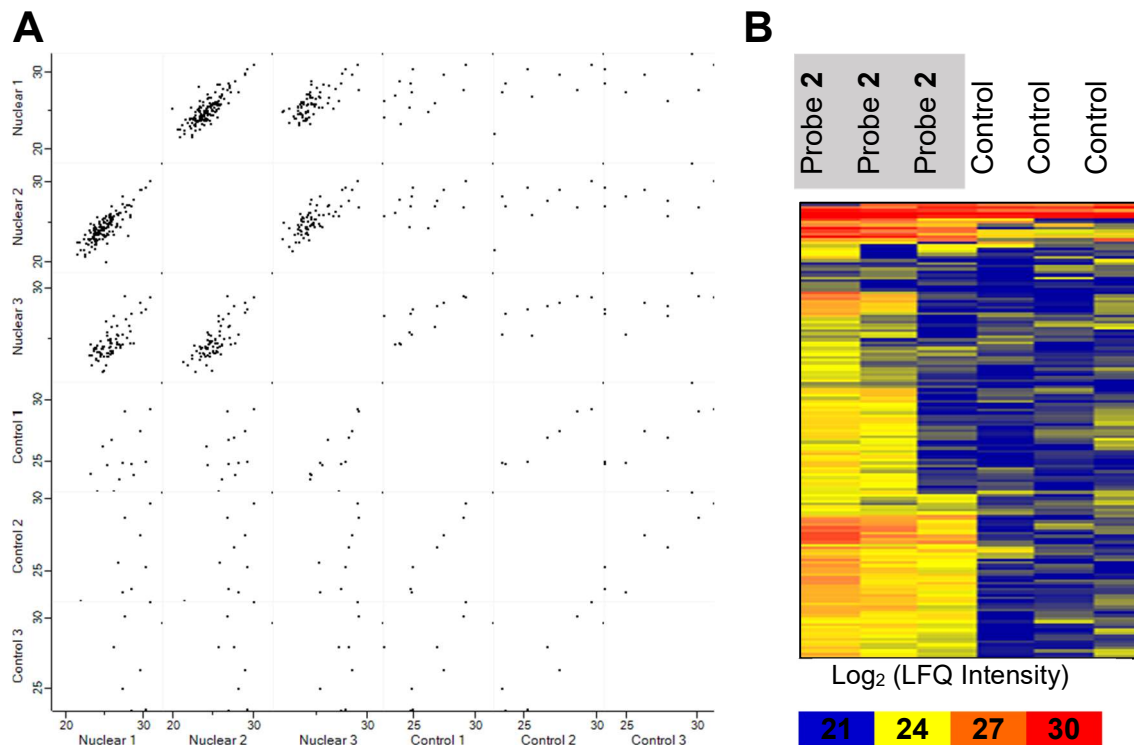


Figure 45: A) Correlation diagrams of the proteins identified by pull-down experiments with AzTB and NeutrAvidin in nuclear extract with probe **2** and DMSO as control. B) Heatmap for all proteins found in the nuclear extract pull-down experiments in terms of their relative abundance $\log_2(\text{LFQ intensity})$ from Euclidian hierarchical row clustering with Perseus 1.5.6.0. High intensity is shown in red and low intensity in blue.

As before, a two-sample student's t-test was carried out to identify statistically significant differences in the populations of the probe samples and the DMSO controls. 33 proteins pulled down from the nuclear extract were significantly enriched by probe **2** compared with the DMSO control (Figure 46).

Of these proteins, four are known to be essential in *P. falciparum* (purple, filled diamonds), whilst twelve are known to be essential in *P. berghei* homologues (green, filled diamonds). Six of these proteins are non-essential, whilst there is no essentiality information about the remainder.

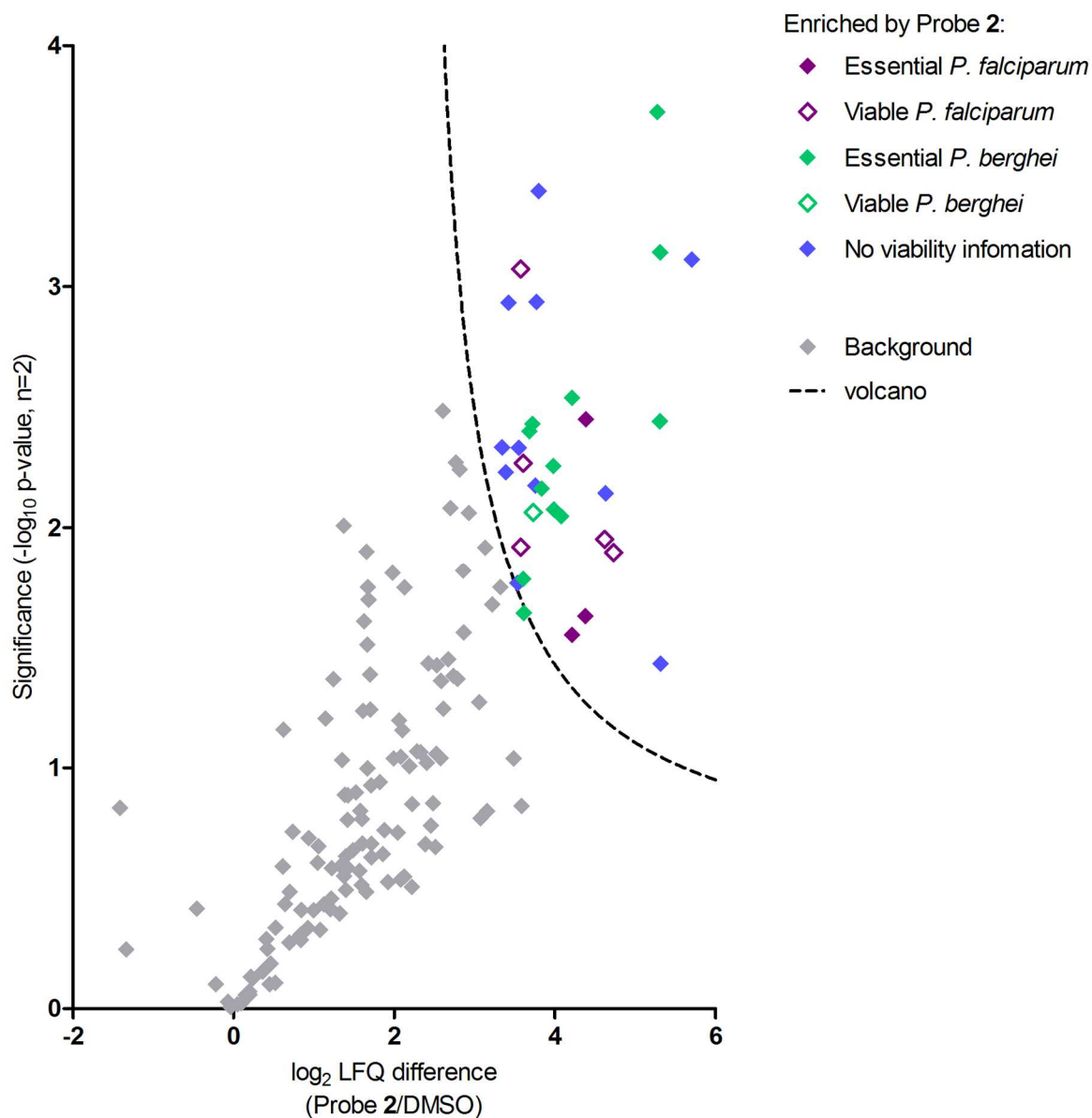


Figure 46: Volcano plot from analysis with Perseus 1.5.6.0 using t-test significance testing of triplicate results with FDR = 0.001 (False Discovery Rate) and $S_0 = 2$. 33 proteins were enriched in the samples containing probe **2** (blue, purple and green).

The 33 enriched proteins from the nuclear extract are listed in Table 9. A number of these proteins are highly abundant, including ribosomal subunits, elongation factors and heat shock proteins, and so these may be non-specific targets being labelled by the probe.

Table 9: The 33 proteins significantly enriched after AzTB and NeutrAvidin pull-down in nuclear extract by probe **2** compared to the DMSO control. Proteins that were also enriched by both probes in the lysate experiments are highlighted in grey.

Gene	Description	Essentiality <i>P. falciparum</i> <i>P. berghei</i>
PF3D7_0905400	high molecular weight rhoptry protein 3	Y
PF3D7_0523000	multidrug resistance protein 1	Y
PF3D7_0929400	high molecular weight rhoptry protein-2	Y
PF3D7_0930300	merozoite surface protein 1	Y
PF3D7_0818200	14-3-3 protein	Y
PF3D7_1408600	40S ribosomal protein S8e, putative	Y
PF3D7_0415900	60S ribosomal protein L15, putative	Y
PF3D7_1246200	actin I	Y
PF3D7_1038000	antigen UB05	Y
PF3D7_1338300	elongation factor 1-gamma, putative	Y
PF3D7_1451100	elongation factor 2	Y
PF3D7_1116800	heat shock protein 101	Y
PF3D7_0917900	heat shock protein 70	Y
PF3D7_0610400	histone H3	Y
PF3D7_1011800	PRE-binding protein	Y
PF3D7_1471100	exported protein 2	Y
PF3D7_0424600	Plasmodium exported protein (PHISTb), unknown function	N
PF3D7_1410400	rhoptry-associated protein 1	N
PF3D7_0102200	ring-infected erythrocyte surface antigen	N
PF3D7_0302500	cytoadherence linked asexual protein 3.1	N
PF3D7_0935800	cytoadherence linked asexual protein 9	N
PF3D7_0708400	heat shock protein 90	N
PF3D7_0422400	40S ribosomal protein S19	?
PF3D7_0307200	60S ribosomal protein L7, putative	?
PF3D7_1130200	60S ribosomal protein P0	?
PF3D7_1037300	ADP/ATP transporter on adenylate translocase	?
PF3D7_1237700	conserved protein, unknown function	?
PF3D7_0818900	heat shock protein 70	?
PF3D7_1352500	thioredoxin-related protein, putative	?

PF3D7_1357000	elongation factor 1-alpha	?
PF3D7_0322900	40S ribosomal protein S3a	?
PF3D7_0807300	Ras-related protein Rab-18	?
PF3D7_0500800	mature parasite-infected erythrocyte surface antigen	?

Interestingly, 9 out of the 33 proteins had been previously found in the lysate experiments using both probe **2** and probe **4**, (highlighted in grey in Table 9) which increases the robustness of the data presented herein. These include transcription factor PREBP, which was pulled-down by probe **2** in the nuclear extract, and by both probes in the lysate, which makes it a potential target for this compound series. To date there are no known inhibitors. Mature parasite-infected erythrocyte surface antigen (MESA), which attaches to the membrane skeleton of infected RBCs,²¹⁴⁻²¹⁶ was also pulled down by the probes in all three experiments. A number of other surface proteins were also identified from the nuclear extract. Several of the other proteins enriched in all three experiments are highly abundant, such as ribosomal proteins and elongation factors, and so these are likely to be non-specific targets. Again, further evidence is needed to identify the specific targets from this data, as only this single experiment has been carried out using the nuclear extract to date.

3.3.5. The *Pf*HKMTs

The *Pf*HKMTs were also absent from the nuclear extract data, in addition to the lysate data. Again, it may be that that these proteins are not the targets of the probes and the diaminoquinazoline series.

However, there may be other reasons these proteins were not identified in the above experiments, such as their low abundance, which could make these kinds of experiments unsuitable for identifying the *Pf*HKMTs. Nevertheless, further attempts may yield results. For example, using lysate or nuclear extract from different stages of the erythrocytic lifecycle

could help if they are more abundant at certain lifecycle stages. Additionally, whole-cell experiments may also be helpful, as this would ensure the proteins were present, folded and active at the incubation part of the protocol for the probes to bind to them.

Besides that, our collaborators at the Pasteur Institute in Paris are working to develop recombinant proteins and antibodies for the *Pf*HKMTs that would allow for direct measurement of the activity of the diminoquinazoline series. However, with the exception of *Pf*SET7,⁷⁰ these are not currently available. Nevertheless, they have managed to raise antibodies for five of the essential *Pf*HKMTs: *Pf*SET1, *Pf*SET3, *Pf*SET6, *Pf*SET7 and *Pf*SET10 (unpublished results), although they have been raised against only weakly active recombinant small fragments of the proteins, and so it is unclear how relevant these are to study the *Pf*HKMTs.

We analysed *Pf*3D7 lysates by Western Blot using the antibodies mentioned above. Although *Pf*SET3, *Pf*SET6, *Pf*SET7 and *Pf*SET10 could be detected (Figure 47), the obtained results are not unambiguous, and a number of other bands can be observed. However, the fact that the antibodies detect these *Pf*HKMTS suggests they could be useful for future analysis after, perhaps, some protocol optimization.

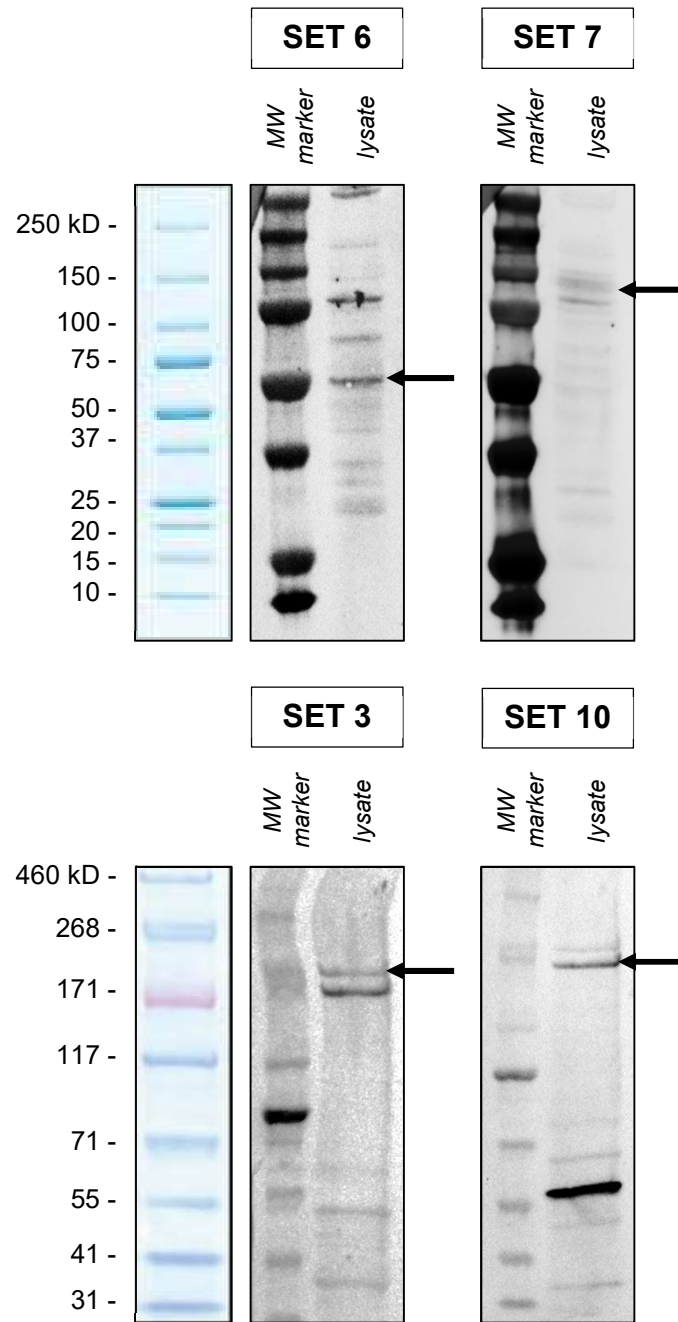


Figure 47: Western blots from *Pf3D7* lysate using antibodies raised against fragments of *PfSET3*, *PfSET6*, *PfSET7* and *PfSET10*. MW means molecular weight. MW markers are shown in blue. Combined chemiluminescence imaging and photograph is shown in grayscale.

PfSET1, on the other hand, is a very large protein (743 kDa), and all Western Blot attempts were unsuccessful. Such large proteins are known to be difficult to run on gels, and also to transfer to the membrane for Western Blot analysis.

Therefore, immunoprecipitation of *Pf*SET1 followed by mass spectrometry analysis was envisioned instead, to see if this HKMT is present in the *Pf*3D7 nuclear extract. This technique relies on the interaction between a given protein and an antibody raised against it, as a means to purify it from a complex sample. In order for this approach to work, the antibody has to be coupled to a solid support that enables its purification afterwards.

Thus, the *Pf*SET1 antibody was first incubated and cross-linked to Magnetic Dynabeads, before incubating the solid-supported antibody with the nuclear extract. These beads were then separated from the protein sample by means of their ability to interact with a magnet, which enables their thorough washing before tryptic digestion and mass spectrometry analysis.²¹⁷ Using this methodology *Pf*SET1 could be identified (Figure 48, pink). However, 964 proteins were identified in total (Figure 48), so the immunoprecipitation was very unclean and requires significant optimisation, including optimising the antibody dilution, introducing more stringent washing and optimising the choice of bead. Replicates would also be needed to obtain statistically significant data.

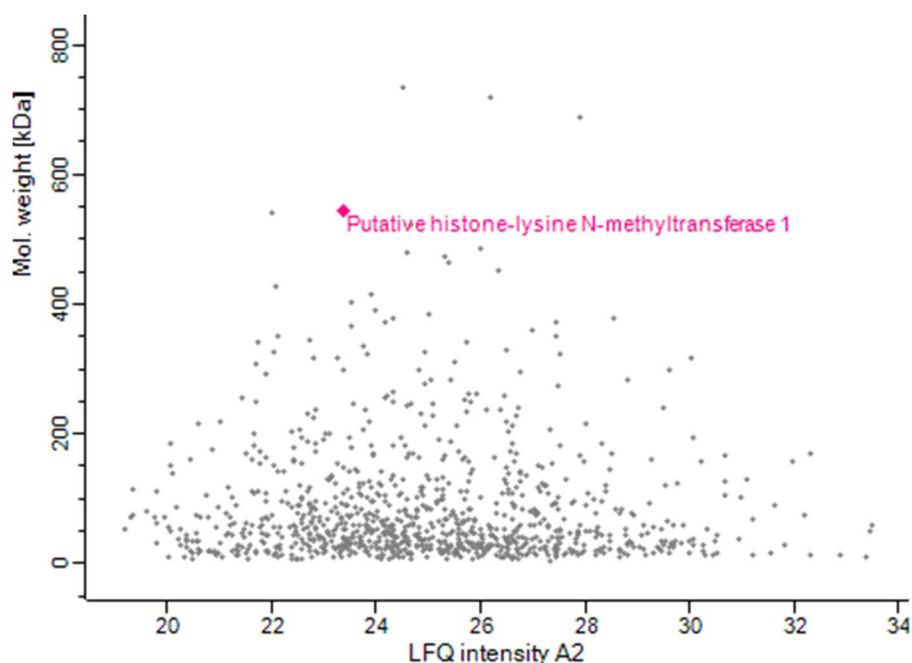


Figure 48: Proteins identified from immunoprecipitation for *Pf*SET1. Immunoprecipitation of labelled nuclear extract using a *Pf*SET1 antibody and Magnetic Protein A Dynabeads followed by trypsin digestion and MS analysis identified 964 proteins including *Pf*SET1 (pink).

Nevertheless, these results suggest this method could be used to enrich lysate for *Pf*SET1 or for the other *Pf*HKMTs, before then carrying out proteomics experiments with the probes, to see if these are indeed targets of the diaminoquinazoline series.

3.4. Conclusions & Future Work

This work provides the tools and protocols for target identification of BIX-01294 and the diaminoquinazoline series: photo-crosslinkable probes have been designed, synthesised, and a protocol developed using in-gel fluorescence. Initial attempts at pull-down proteomics have allowed the identification of a number of proteins significantly enriched by the probes compared to the control samples, although further work is needed to identify and validate the targets, and to further investigate whether the *Pf*HKMTs are targeted by this series.

For the identification of specific and non-specific targets, competition proteomics experiments would be very valuable as shown by in-gel fluorescence experiments. For specific targets, the LFQ intensity of the peptides should be reduced when the proteins are pre-incubated with BIX-01294 or an active inhibitor when compared with probe samples, and therefore NeutrAvidin pull-down and proteomics analysis should therefore allow for the identification of these specific targets. In addition, an inactive compound from the series could be selected and transformed into a probe. This “inactive probe” will still label the high abundance non-specific proteins but won’t label the specific targets accountable for antiparasitic activity.

The asexual erythrocytic lifecycle of *P. falciparum* is complex, with different proteins expressed at different blood stages. The diaminoquinazoline series are potent at all blood cycle stages, and so now the protocols have been developed, it would be interesting to try these proteomics experiments with synchronised parasites from the different lifecycle stages, and compare the proteins identified.

Finally, whole-cell experiments, with probe incubation and photo-crosslinking taking place before cell lysis, could provide data more relevant to the *in vivo* effects of these compounds, and would guarantee that proteins are in a folded, active state.

If recombinant *PF*HKMTs can be expressed and purified, this will provide the most direct and unbiased method to validate these as targets of the diaminoquinazoline series. Alternatively, further work can be done to attempt to enrich the samples for these proteins, including working with nuclear extract, and perform immunoprecipitations with the antibodies.

Overall, the work presented herein provides a solid foundation for future development. This work sets the basis for further efforts on target identification and validation, with the final goal of uncovering the mechanism of action of the diaminoquinazoline series, towards their use as novel antimalarials.

4. Experimental

4.1. Chemical Synthesis

4.1.1. General Procedures

All reactions were performed under an atmosphere of dry nitrogen. Room temperature (rt) refers to ambient temperature. Temperatures of 0 °C were achieved using an ice-water bath, whilst lower temperatures were achieved using an acetone-dry ice bath. Reagents and solvents were supplied from commercial sources and used as supplied. Where anhydrous conditions were required, dichloromethane (DCM), triethylamine, and pyridine were distilled over calcium hydride, methanol was distilled over magnesium, and other anhydrous chemicals were obtained commercially. Column chromatography was carried out using Fluorochem 60 silica gel (230–400 mesh, 40–63 µm). Thin layer chromatography (TLC) was performed on aluminium plates using Merck Kiesegel 60 F254 (230–400 mesh). Fluorescent treated silica were visualised under UV (254 nm), or by staining with potassium permanganate or ninhydrin solutions. Solvents were removed by rotary evaporator at 40 °C or below and the compounds further dried under high vacuum. Microwave irradiation was carried out using a Discoverer SP system (CEM Technology). ¹H, ¹³C and ¹⁹F NMR (nuclear magnetic resonance) were recorded on a Bruker Advance 400 spectrophotometer at 400 MHz, 100 MHz and 471 MHz respectively, or ¹³C NMR were recorded on a Bruker Advance 500 spectrophotometer at 125 MHz. Chemical shifts are referenced to the residual solvent peak. Proton spectra (δH) are quoted in parts per million (ppm) to the nearest 0.01 ppm. Coupling constants (*J*) are reported in Hertz (Hz) to the nearest 0.1 Hz. Data are reported as follows: chemical shift, multiplicity (s, singlet; d, doublet; t, triplet; m, multiplet; or as a combination of these), coupling constant(s), integration and assignment. Carbon spectra were recorded with broadband proton spin decoupling and chemical shifts (δC) are quoted in ppm to the nearest 0.1 ppm. Coupling constants (*J*) to ¹⁹F are reported in Hz to the nearest 1 Hz. Fluorine spectra (δF) are quoted in parts per million (ppm) to the nearest 0.1

ppm. Melting points were obtained on a Reichert-Thermovar melting point apparatus and are uncorrected. Infrared spectra were recorded on a Perkin Elmer Frontier FT-IR Spectrometer. Reported absorptions are given in wavenumbers (cm^{-1}). High resolution mass spectra were recorded by the Imperial College London Department of Chemistry Mass Spectroscopy Service using a Micromass Autospec Premier and Micromass LCT Premier spectrometer. Mass values are quoted within the error limits of ± 5 ppm mass units. ESI refers to electrospray mass ionisation and EI to electron impact.

To assess the purity of the product, LC-MS analysis was carried out by the Imperial College London Department of Chemistry Mass Spectroscopy Service using a Waters Aquity UPLC I-CLASS instrument. A Waters BEH Acquity C18 column (50mm x 2.1mm) was used with a column temperature of 40 °C, an injection volume of 10 μL and a flow rate of 0.5 mL min^{-1} . Starting at 95% solvent A (99.9% water, 0.1% formic acid) and 5% solvent B (99.9% acetonitrile, 0.1% formic acid), a linear gradient to 5% solvent A and 95% solvent B at 3.2 minutes was used. The system was returned to 95% solvent A and 5% solvent B at 3.5 minutes with a total run time of 4 minutes. The purity was assessed using the UV trace, using a Photodiode Array Detector (210 – 280 nm). All solvents used were HPLC grade (Sigma Aldrich or Thermo Fisher).

General Procedure A: Position 4 Substitution

6,7-dimethoxy-2,4-dichloroquinazoline (1 equiv) was dissolved in anhydrous tetrahydrofuran (THF). Amine (1 equiv) in anhydrous THF was added, followed by *N,N*-diisopropylethylamine (DIPEA) (3 equiv). The reaction mixture was allowed to stir at 30 °C for 20 h. The reaction mixture was then concentrated *in vacuo* and the residue dissolved in DCM. The solution was washed with an equal volume of water and brine and the organic layer dried over magnesium sulfate, concentrated *in vacuo* and purified by column chromatography.

General Procedure B: Position 2 Substitution Method 1

The 6,7-dimethoxy-2-amino-4-chloroquinazoline (1 equiv) and the amine (3 equiv) were dissolved in toluene and the reaction mixture heated to 130 °C for 1-2 h under microwave irradiation. The reaction mixture was dissolved in DCM, washed with an equal volume of water and dried over magnesium sulfate, concentrated *in vacuo* and purified by column chromatography.

General Procedure C: Position 2 Substitution Method 2

The 6,7-dimethoxy-2-amino-4-chloroquinazoline (1 equiv) and the amine (3 equiv) were heated to 130 °C for 1 h under microwave irradiation. The reaction mixture was dissolved in DCM, washed with an equal volume of water and dried over magnesium sulfate, concentrated *in vacuo* and purified by column chromatography.

General Procedure D: Reductive Amination

The ketone (1 equiv) in 1,2-dichloroethane was treated with 4-(*N*-Boc-amino)-piperidine (1.5 equiv) and the mixture allowed to stir at rt for 3 h. Sodium triacetoxyborohydride ($\text{NaBH}(\text{OAc})_3$) (1.4 equiv) was then added over 30 min, and the solution was left to stir for 18 h. The reaction mixture was quenched using saturated sodium bicarbonate solution and extracted with an equal volume of ethyl acetate (EtOAc). The aqueous layer was further extracted twice with EtOAc and the combined organic layers were dried over magnesium sulfate, concentrated *in vacuo* and purified by column chromatography.

General Procedure E: Boc Deprotection

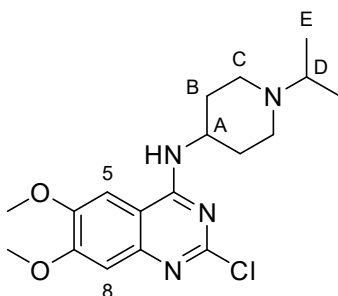
The Boc-protected amine was dissolved in DCM. An equal volume of TFA was added dropwise, and the mixture left to stir at rt for 4 h. The mixture was then basified with NaOH solution (2M) to pH 12 and extracted with DCM. The organic layers were combined and concentrated *in vacuo*.

General Procedure F: Nitrile Hydrolysis

A solution of the benzonitrile 2,4-diaminoquinazoline (1 equiv) and potassium carbonate (0.2 equiv) in water was heated to 150 °C for 1 h under microwave irradiation. The reaction mixture was diluted with water and then extracted with an equal volume of DCM. The organic layer was washed with brine, dried over magnesium sulfate, concentrated *in vacuo* and purified by column chromatography.

4.1.2. Expanding the SAR: Improving Pharmacodynamic Properties

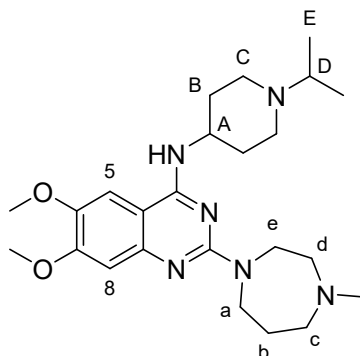
Compound 17a: 2-chloro-*N*-(1-isopropylpiperidin-4-yl)-6,7-dimethoxyquinazolin-4-amine



Prepared by general procedure **A** with 6,7-dimethoxy-2,4-dichloroquinazoline (518 mg, 2.0 mmol), 1-isopropyl-piperidin-4-ylamine (284 mg, 2.0 mmol) and DIPEA (1.1 mL, 6.0 mmol). The crude product was purified by column chromatography eluting with a gradient of DCM/NH₃ in MeOH (7N) (100:0 → 95:5) to yield the product as a pale-yellow solid (352 mg, 48%).

R_f 0.52 (DCM/NH₃ in MeOH (7N), 97:3). **¹H NMR** (400 MHz, CDCl₃) δ 7.16 (s, 1H, **5**), 6.81 (s, 1H, **8**), 5.38 (d, *J* = 7.8, 1H, **NH**), 4.37 – 4.25 (m, 1H, **A**), 4.01 (s, 3H, **OCH₃**), 4.04 (s, 3H, **OCH₃**), 3.04 – 2.93 (m, 2H, **C** eq), 2.86 (p, *J* = 6.6, 1H, **D**), 2.51 – 2.41 (m, 2H, **C** ax), 2.22 (d, *J* = 12.4, 2H, **B** eq), 1.74-1.62 (m, 2H, **B** ax), 1.12 (d, *J* = 6.6, 6H, **E**). **¹³C NMR** (101 MHz, CDCl₃) δ 159.1, 156.2, 155.0, 149.2, 148.1, 107.4, 106.7, 99.5, 56.4, 56.3, 54.7, 48.5, 47.6 (2C), 32.4 (2C), 18.3 (2C). **IR** (neat): 3245, 2925. **HRMS** (ESI): *m/z* found [M+H]⁺ 365.1745, C₁₈H₂₆N₄O₂Cl requires 365.1744.

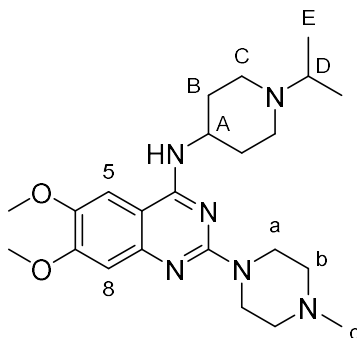
Compound 1: *N*-(1-isopropylpiperidin-4-yl)-6,7-dimethoxy-2-(4-methyl-1,4-diazepan-1-yl)-quinazolin-4-amine



Prepared by general procedure **B** with compound **17a** (30 mg, 0.082 mmol) and 1-methylhomopiperazine (0.030 mL, 0.24 mmol). The crude product was purified by column chromatography eluting with a gradient of DCM/NH₃ in MeOH (7N) (100:0 → 90:10) to yield the product as a yellow solid (8 mg, 22%) which was ≥95% pure by LC-MS analysis.

R_f 0.16 (DCM/NH₃ in MeOH (7N), 95:5). **m.p.** 71-74 °C. **¹H NMR** (400 MHz, CDCl₃): δ 6.92 (s, 1H, **5**), 6.71 (s, 1H, **8**), 4.96 (d, *J* = 7.2, 1H, **NH**), 4.21-4.08 (m, 1H, **A**), 4.03 – 3.99 (m, 2H, **e**), 3.98 (s, 3H, **OCH₃**), 3.96 (s, 3H, **OCH₃**), 3.91 (t, 6.4, 2H, **a**), 3.02 – 2.91 (m, 2H, **C** eq), 2.81 (p, *J* = 6.6, 1H, **D**), 2.77 – 2.70 (m, 2H, **c**), 2.65 – 2.57 (m, 2H, **d**), 2.41 (s, 3H, **c**), 2.39 – 2.31 (m, 2H, **C** ax), 2.26 – 2.17 (m, 2H, **B** eq), 2.10 – 2.00 (m, 2H, **b**), 1.70 – 1.56 (m, 2H, **B** ax), 1.12 (d, *J* = 6.6, 6H, **E**). **¹³C NMR** (101 MHz, CDCl₃) δ 158.5, 158.0, 154.4, 149.4, 145.1, 106.0, 102.7, 100.7, 58.9, 57.4, 56.4, 56.0, 54.8, 48.5, 47.8 (2C), 46.7, 45.9, 45.7, 32.4 (2C), 27.8, 18.4 (2C). **IR** (neat): 3326, 2934. **HRMS** (ESI): *m/z* found [M+H]⁺ 443.3139, C₂₄H₃₉N₆O₂ requires 443.3134.

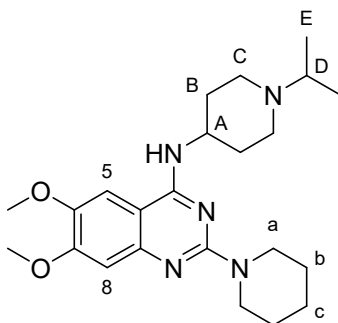
Compound 2: *N*-(1-isopropylpiperidin-4-yl)-6,7-dimethoxy-2-(4-methylpiperazin-1-yl)-quinazolin-4-amine



Prepared by general procedure **B** with compound **17a** (30 mg, 0.082 mmol) and 1-methylpiperazine (0.030 mL, 0.27 mmol). The crude product was purified by column chromatography eluting with a gradient of DCM/NH₃ in MeOH (7N) (99:1 → 95:5) to yield the product as a yellow solid (9 mg, 26%) which was ≥95% pure by LC-MS analysis.

R_f 0.13 (DCM/NH₃ in MeOH (7N), 95:5). **m.p.** 73-77 °C. **¹H NMR** (400 MHz, CDCl₃) δ 6.93 (s, 1H, **5**), 6.71 (s, 1H, **8**), 4.99 (d, *J* = 7.2, 1H, **NH**), 4.30 – 4.06 (m, 1H, **A**), 3.99 (s, 3H, **OCH₃**), 3.96 (s, 3H, **OCH₃**), 3.89 (t, *J* = 5.0, 4H, **a**), 2.83 (p, *J* = 6.5, 1H, **D**), 2.52 (t, *J* = 5.0, 4H, **b**), 2.44 – 2.38 (m, 2H, **C** eq), 2.37 (s, 3H, **c**), 2.26 – 2.18 (m, 2H, **C** ax), 1.69 – 1.58 (m, 2H, **B** eq), 1.31 – 1.23 (m, 2H, **B** ax), 1.12 (d, *J* = 6.5, 6H, **E**). **¹³C NMR** (101 MHz, CDCl₃) δ 158.8, 152.7, 151.9, 150.8, 145.6, 106.2, 103.1, 100.6, 93.4, 56.4, 56.0, 55.3 (2C), 54.6, 47.8 (2C), 46.4, 44.0 (2C), 32.6 (2C), 18.5 (2C). **IR** (neat): 3303, 2927. **HRMS** (ESI): *m/z* found [M+H]⁺ 429.2981, C₂₃H₃₇N₆O₂ requires 429.2978.

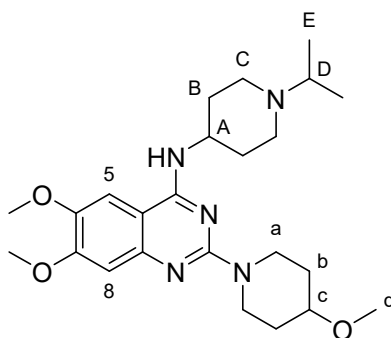
Compound 3: *N*-(1-isopropylpiperidin-4-yl)-6,7-dimethoxy-2-(piperidin-1-yl)-quinazolin-4-amine



Prepared by general procedure **B** with compound **17a** (30 mg, 0.082 mmol) and piperidine (0.025 mL, 0.25 mmol). The crude product was purified by column chromatography eluting with DCM/NH₃ in MeOH (7N) (98:2) to yield the product as a yellow solid (6 mg, 18%) which was ≥95% pure by LC-MS analysis.

R_f 0.28 (DCM/NH₃ in MeOH (7N), 95:5). **m.p.** 82–84 °C. **¹H NMR** (400 MHz, CDCl₃) δ 6.92 (s, 1H, **5**), 6.70 (s, 1H, **8**), 4.94 (d, *J* = 7.2, 1H, **NH**), 4.18 – 4.08 (m, 1H, **A**), 3.98 (s, 3H, **OCH₃**), 3.97 (s, 3H, **OCH₃**), 3.84 (d, *J* = 5.9, 4H, **a**), 2.95 (d, *J* = 11.8, 2H, **C** eq), 2.81 (p, *J* = 6.5, 1H, **D**), 2.49 – 2.32 (m, 2H, **C** ax), 2.23 (d, *J* = 13.6, 2H, **B** eq), 1.75 – 1.53 (m, 8H, **B** ax, **b**, **c**), 1.11 (d, *J* = 6.5, 6H, **E**). **¹³C NMR** (101 MHz, CDCl₃) δ 158.2, 154.3, 154.0, 146.9, 145.2, 106.1, 102.8, 100.6, 56.4, 56.0, 54.7, 48.4, 47.8 (2C), 45.1 (2C), 32.5 (2C), 26.0 (2C), 25.1, 18.5 (2C). **IR** (neat): 3398, 2930. **HRMS** (ESI): *m/z* found [M+H]⁺ 414.2861, C₂₃H₃₆N₅O₂ requires 414.2869.

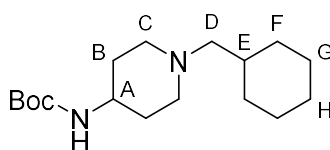
Compound 4: *N*-(1-isopropylpiperidin-4-yl)-6,7-dimethoxy-2-(4-methoxypiperidin-1-yl)-quinazolin-4-amine



Prepared by general procedure **C** with compound **17a** (30 mg, 0.082 mmol) and 4-methoxypiperidine (30 mg, 0.26 mmol). The crude product was purified by column chromatography eluting with a gradient of DCM/NH₃ in MeOH (7N) (99:1 → 95:5) to yield the product as a yellow solid (5 mg, 14%) which was ≥95% pure by LC-MS analysis.

R_f 0.33 (DCM/NH₃ in MeOH (7N), 97.5:2.5). **m.p.** 72-75 °C. **¹H NMR** (400 MHz, CDCl₃) δ 6.92 (s, 1H, **5**), 6.70 (s, 1H, **8**), 4.96 (d, *J* = 7.3, 1H, **NH**), 4.56 – 4.42 (m, 2H, **a** eq), 4.14 (d, *J* = 6.9, 1H, **A**), 3.99 (s, 3H, OCH₃), 3.97 (s, 3H, OCH₃), 3.48 – 3.45 (m, 1H, **c**), 3.43 (s, 3H, **d**), 3.31 (m, 2H, **a** ax), 2.96 (d, *J* = 11.5, 2H, **C** eq), 2.86 – 2.77 (m, 1H, **D**), 2.44 – 2.33 (m, 2H, **C** ax), 2.23 (d, *J* = 12.6, 2H, **B** eq), 2.02 (d, *J* = 12.6, 2H, **B** ax), 1.67 – 1.55 (m, 4H, **b**), 1.28 (s, 1H), 1.12 (d, *J* = 6.5, 6H, **E**). **¹³C NMR** (101 MHz, CDCl₃) δ 154.4, 153.3, 149.4, 146.8, 145.4, 106.1, 103.0, 100.6, 77.2, 56.4, 56.0, 55.6, 54.7, 48.4, 47.6 (2C), 41.8 (2C), 32.5 (2C), 30.9 (2C), 18.4 (2C). **IR** (neat): 3319, 2932. **HRMS** (ESI): *m/z* found [M+H]⁺ 444.2972, C₂₄H₃₈N₅O₃ requires 444.2975.

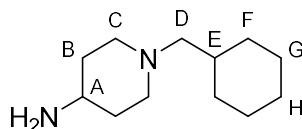
Compound 18b: *tert*-butyl 1-(cyclohexylmethyl)-piperidin-4-ylcarbamate



Prepared by general procedure **D** with 4-(*N*-Boc-amino)-piperidine (901 mg, 4.5 mmol), NaBH(OAc)₃ (890 mg, 4.2 mmol) and cyclohexanecarboxaldehyde (0.36 mL, 3.0 mmol). The crude product was purified by column chromatography eluting with DCM/NH₃ in MeOH (7N) (95:5) to yield the product as a white solid (856 mg, 96%).

R_f 0.23 (EtOAc/pentane, 70:30). **¹H NMR** (400 MHz, CDCl₃) δ 4.43 (s, 1H, **A**), 3.47 (s, 1H, **NH**), 2.78 (d, *J* = 11.6, 2H, **C** eq), 2.10 (d, *J* = 7.1, 2H, **D**), 2.06 – 1.96 (m, 2H, **C** ax), 1.95 – 1.86 (m, 2H, **B** eq), 1.82 – 1.57 (m, 5H, **B** ax, **E**, **F** eq), 1.47 (s, 9H, Boc (**CH₃**)₃), 1.45 – 1.11 (m, 6H, **F** ax, **G**), 1.00-0.78 (m, 2H, **H**). **HRMS** (ESI): *m/z* found [M+H]⁺ 297.2542, C₁₇H₃₃N₂O₂ requires 297.2542.

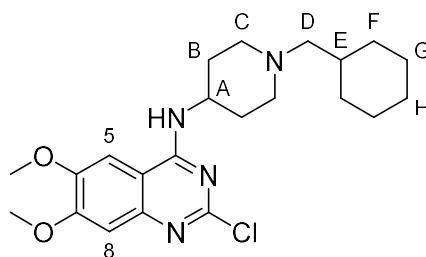
Compound 19b: 1-(cyclohexylmethyl)-piperidin-4-amine



Prepared by general procedure **E** with compound **18b** (800 mg, 2.7 mmol), the product was collected as a brown oil (513 mg, 97%).

¹H NMR (400 MHz, CDCl₃) δ 2.91 – 2.78 (m, 2H, **C** eq), 2.67 (tt, *J* = 10.4, 4.2, 1H, **A**), 2.14 (d, *J* = 7.0, 2H, **D**), 2.05 – 1.92 (m, 2H, **C** ax), 1.87 – 1.63 (m, 8H, **B**, **F**), 1.56 – 1.34 (m, 3H, **E**, **G** eq), 1.32 – 1.11 (m, 2H, **G** ax), 0.96 – 0.79 (m, 2H, **H**). **HRMS** (ESI): *m/z* found [M+H]⁺ 197.2022, C₁₂H₂₅N₂ requires 197.2018.

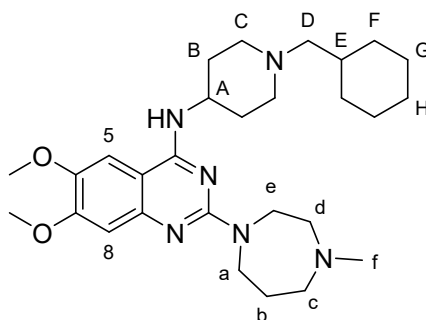
Compound 17b: 2-chloro-*N*-(1-(cyclohexylmethyl)-piperidin-4-yl)-6,7-dimethoxyquinazolin-4-amine



Prepared by general procedure **A** with 6,7-dimethoxy-2,4-dichloroquinazoline (554 mg, 2.1 mmol), compound **19b** (420 mg, 2.1 mmol) and DIPEA (1.2 mL, 6.6 mmol). The crude product was purified by column chromatography eluting with a gradient of DCM/NH₃ in MeOH (7N) (100:0 → 92:8) to yield the product as a pale-yellow solid (512 mg, 41%).

*R*_f 0.60 (DCM/NH₃ in MeOH (7N), 95:5). **¹H NMR** (400 MHz, CDCl₃) δ 7.17 (s, 1H, **5**), 6.77 (s, 1H, **8**), 5.29 (d, *J* = 7.6, 1H, **NH**), 4.32 (s, 1H, **A**), 4.04 (s, 3H, **OCH₃**), 4.01 (s, 3H, **OCH₃**), 2.98 – 2.86 (m, 2H, **C** eq), 2.29 – 2.12 (m, 6H, **C** ax, **D**, **B** eq), 1.89 – 1.63 (m, 7H, **B** ax, **e**, **F**), 1.36 – 1.16 (m, 4H, **G**), 1.01 – 0.84 (m, 2H, **H**). **¹³C NMR** (101 MHz, CDCl₃) δ 159.1, 156.4, 155.0, 149.0, 148.1, 110.0, 107.4, 99.5, 65.6, 56.4, 56.3, 52.9, 48.3, 35.3 (2C), 32.1 (2C), 32.0 (2C), 26.8, 26.2 (2C). **IR** (neat): 3229, 2917. **HRMS** (ESI): *m/z* found [M+H]⁺ 419.2214, C₂₂H₃₂N₄O₂Cl requires 419.2214.

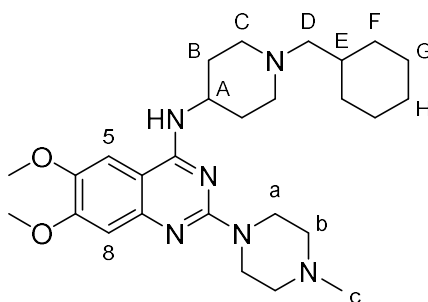
Compound 5: *N*-(1-(cyclohexylmethyl)-piperidin-4-yl)-6,7-dimethoxy-2-(4-methyl-1,4-diazepan-1-yl)-quinazolin-4-amine



Prepared by general procedure **B** with compound **17b** (30 mg, 0.072 mmol) and 1-methylhomopiperazine (0.030 mL, 0.24 mmol). The crude product was purified by column chromatography eluting with a gradient of DCM/NH₃ in MeOH (7N) (100:0 → 90:10) to yield the product as a yellow solid (23 mg, 64%) which was ≥95% pure by LC-MS analysis.

R_f 0.48 (DCM/NH₃ in MeOH (7N), 97.5:2.5). **m.p.** 116-118 °C. **¹H NMR** (400 MHz, CDCl₃) δ 6.91 (s, 1H, **5**), 6.71 (s, 1H, **8**), 4.96 (d, *J* = 7.2, 1H, **NH**), 4.18 – 4.03 (m, 1H, **A**), 4.02 – 3.99 (m, 2H, **e**), 3.98 (s, 3H, **OCH₃**), 3.96 (s, 3H, **OCH₃**), 3.90 (t, *J* = 6.4, 2H, **a**), 2.90 (d, *J* = 11.7, 2H, **C** eq), 2.79 – 2.70 (m, 2H, **c**), 2.64 – 2.56 (m, 2H, **d**), 2.40 (s, 3H, **f**), 2.22 – 1.96 (m, 8H, **C** ax, **D**, **B** eq, **b**), 1.86 – 1.57 (m, 6H, **B** ax, **F**), 1.58 – 1.45 (m, 1H, **E**), 1.34 – 1.15 (m, 4H, **G**), 0.99 – 0.83 (m, 2H, **H**). **¹³C NMR** (101 MHz, CDCl₃) δ 158.5, 158.0, 154.3, 149.4, 145.0, 106.0, 102.8, 100.7, 65.8, 58.9, 57.3, 56.4, 56.0, 53.2 (2C), 48.5, 46.7, 45.9, 45.7, 35.4, 32.3 (2C), 32.0 (2C), 27.8, 26.8, 26.2 (2C). **IR** (neat): 3320, 2921. **HRMS** (ESI): *m/z* found [M+H]⁺ 497.3593 C₂₈H₄₅N₆O₃ requires 497.3604.

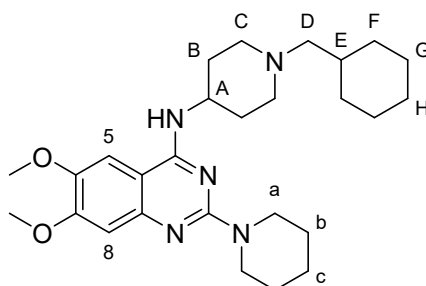
Compound 6: *N*-(1-(cyclohexylmethyl)-piperidin-4-yl)-6,7-dimethoxy-2-(4-methylpiperazin-1-yl)-quinazolin-4-amine



Prepared by general procedure **C** with compound **17b** (30 mg, 0.072 mmol) and 1-methylpiperazine (0.025 mL, 0.23 mmol). The crude product was purified by column chromatography eluting with a gradient of DCM/NH₃ in MeOH (7N) (100:0 → 90:10) to yield the product as a yellow solid (7 mg, 20%) which was ≥95% pure by LC-MS analysis.

R_f 0.51 (DCM/NH₃ in MeOH (7N), 97.5:2.5). **m.p.** 97-100 °C. **¹H NMR** (400 MHz, CDCl₃) δ 6.92 (s, 1H, **5**), 6.71 (s, 1H, **8**), 5.00 (d, *J* = 7.3, 1H, **NH**), 4.22 – 4.08 (m, 1H, **A**), 3.98 (s, 3H, **OCH₃**), 3.96 (s, 3H, **OCH₃**), 3.93 – 3.85 (m, 4H, **a**), 2.89 (d, *J* = 12.1, 2H, **C** eq), 2.52 (t, *J* = 5.0, 4H, **b**), 2.37 (s, 3H, **c**), 2.19 (s, 2H, **D**), 2.19 – 2.07 (m, 4H, **C** ax, **B** eq), 1.87 – 1.57 (m, 6H, **B** ax, **F**), 1.57 – 1.44 (m, 1H, **E**), 1.32 – 1.16 (m, 4H, **G**), 0.98 – 0.82 (m, 2H, **H**). **¹³C NMR** (101 MHz, CDCl₃) δ 158.9, 158.2, 154.3, 149.2, 145.4, 106.2, 103.2, 100.5, 65.8, 56.3, 56.0, 55.3 (2C), 53.2 (2C), 48.4, 46.4, 44.0 (2C), 35.4, 32.3 (2C), 32.0 (2C), 26.8, 26.2 (2C). **IR** (neat): 3329, 2922. **HRMS** (ESI): *m/z* found [M+H]⁺ 483.3449, C₂₇H₄₃N₆O₂ requires 483.3448.

Compound 7: *N*-(1-(cyclohexylmethyl)-piperidin-4-yl)-6,7-dimethoxy-2-(piperidin-1-yl)-quinazolin-4-amine

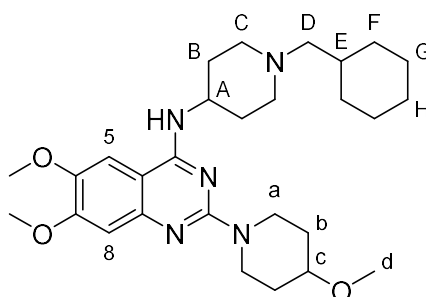


Prepared by general procedure **B** with compound **17b** (30 mg, 0.072 mmol) and piperidine (0.025 mL, 0.25 mmol). The crude product was purified by column chromatography eluting with a gradient of DCM/NH₃ in MeOH (7N) (100:0 → 90:10) to yield the product as a pale orange-brown solid (19 mg, 53%) which was ≥95% pure by LC-MS analysis.

R_f 0.67 (DCM/NH₃ in MeOH (7N), 97:3). **m.p.** 79-82 °C. **¹H NMR** (400 MHz, MeOH-d₄) δ 6.98 (s, 1H, **5**), 6.71 (s, 1H, **8**), 5.03 (s, 1H, **NH**), 4.21 – 4.10 (m, 1H, **A**), 3.94 (s, 3H, **OCH₃**), 3.92 (s, 3H, **OCH₃**), 3.91-3.76 (m, 4H, **a**), 2.92 (d, *J* = 11.3, 2H, **C** eq), 2.27 – 2.07 (m, 6H, **C** ax, **D**, **B** eq), 1.86 – 1.56 (m, 12H, **B** ax, **F**, **b**, **c**), 1.59 – 1.47 (m, 1H, **E**), 1.39 – 1.12 (m, 4H,

G), 1.01 – 0.81 (m, 2H, **H**). ^{13}C NMR (101 MHz, CDCl_3) δ 158.2, 156.3, 154.4, 151.3, 145.3, 105.7, 102.8, 100.7, 65.7, 56.4, 56.1, 53.2 (2C), 48.3, 45.2 (2C), 35.4, 32.1 (2C), 32.0 (2C), 26.8, 26.2 (2C), 26.0 (2C), 25.1. IR (neat): 3398, 2922. HRMS (ESI): m/z found $[\text{M}+\text{H}]^+$ 468.3328, $\text{C}_{27}\text{H}_{42}\text{N}_5\text{O}_3$ requires 468.3339.

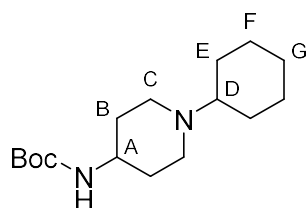
Compound 8: *N*-(1-(cyclohexylmethyl)-piperidin-4-yl)-6,7-dimethoxy-2-(4-methoxypiperidin-1-yl)-quinazolin-4-amine



Prepared by general procedure **C** with compound **17b** (30 mg, 0.072 mmol) and 4-methoxypiperidine (25 mg, 0.22 mmol). The crude product was purified by column chromatography eluting with a gradient of DCM/NH_3 in MeOH (7N) (99:1 $\rightarrow$ 95:5) to yield the product as a pale brown solid (15 mg, 42%) which was $\geq 95\%$ pure by LC-MS analysis.

R_f 0.53 (DCM/NH_3 in MeOH (7N), 97.5:2.5). **m.p.** 67-70 $^\circ\text{C}$. ^1H NMR (400 MHz, CDCl_3) δ 6.92 (s, 1H, **5**), 6.72 (s, 1H, **8**), 5.01 (d, $J = 7.2$, 1H, **NH**), 4.50 – 4.44 (m, 2H, **a** eq), 4.19 – 4.09 (m, 1H, **A**), 3.98 (s, 3H, **OCH₃**), 3.95 (s, 3H, **OCH₃**), 3.50 – 3.44 (m, 1H, **c**), 3.42 (s, 3H, **d**), 3.36 – 3.27 (m, 2H, **a** ax), 2.96 – 2.83 (m, 2H, **C** eq), 2.27 – 2.08 (m, 8H, **C** ax, **D**, **b**), 2.04 – 1.98 (m, 2H, **B** eq), 1.87 – 1.43 (m, 7H, **B** ax, **E**, **F**), 1.41 – 1.08 (m, 4H, **G**), 0.95 – 0.85 (m, 2H, **H**). ^{13}C NMR (101 MHz, CDCl_3) δ 158.8, 158.3, 154.3, 149.3, 145.3, 106.1, 103.0, 100.6, 77.2, 65.8, 56.3, 56.0, 55.6, 53.2 (2C), 48.4, 41.8 (2C), 35.4, 32.2 (2C), 32.0 (2C), 30.9 (2C), 26.8, 26.2 (2C). IR (neat): 3397, 2922. HRMS (ESI): m/z found $[\text{M}+\text{H}]^+$ 498.3451, $\text{C}_{28}\text{H}_{44}\text{N}_5\text{O}_3$ requires 498.3444.

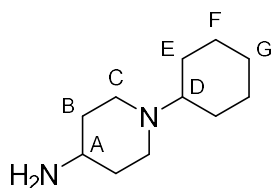
Compound 18c: *tert*-butyl 1-cyclohexylpiperidin-4-ylcarbamate



Prepared by general procedure **D** with 4-(*N*-Boc-amino)-piperidine (901 mg, 4.5 mmol), NaBH(OAc)₃ (890 mg, 4.2 mmol), and cyclohexanone (0.31 mL, 3.0 mmol), with the addition of acetic acid (0.20 mL). The crude product was purified by column chromatography eluting with DCM/NH₃ in MeOH (7N) (95:5) to yield the product as a white solid (416 mg, 49%).

¹H NMR (400 MHz, CDCl₃) δ 4.44 (s, 1H, NH), 3.50 – 3.36 (m, 1H, **A**), 2.95 – 2.78 (m, 2H, **C** eq), 2.41 – 2.20 (m, 3H, **C** ax, **D**), 1.96 (d, J = 12.3, 2H, **B** eq), 1.91 – 1.74 (m, 4H, **B** ax, **E** eq), 1.64 (d, J = 13.0, 2H, **E** ax), 1.47 (s, 9H, Boc (CH₃)₃), 1.44 – 1.35 (m, 2H, **F** eq), 1.32 – 1.17 (m, 4H, **F** ax, **G**).

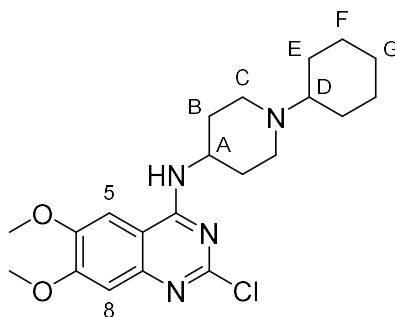
Compound 19c: 1-cyclohexylpiperidin-4-amine



Prepared by general procedure **E** with compound **18c** (390 mg, 1.4 mmol), the product was collected as a brown oil (236 mg, 94%).

¹H NMR (400 MHz, CDCl₃) δ 2.89 (d, J = 11.3, 2H, **C** eq), 2.68 – 2.57 (m, 1H, **A**), 2.37 – 2.20 (m, 3H, **C** ax, **D**), 1.94 – 1.74 (m, 6H, **B**, **E** eq), 1.46 – 1.32 (m, 2H, **E** ax), 1.30 – 1.18 (m, 6H, **F**, **G**).

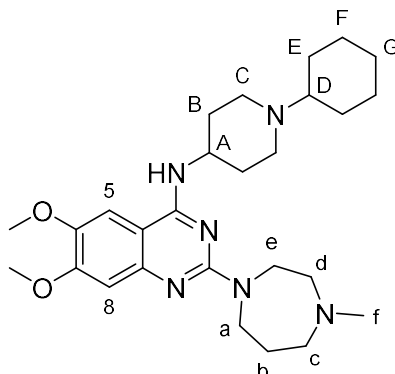
Compound 17c: 2-chloro-*N*-(1-cyclohexylpiperidin-4-yl)-6,7-dimethoxyquinazolin-4-amine



Prepared by general procedure **A** with 6,7-dimethoxy-2,4-dichloroquinazoline (321 mg, 1.2 mmol), compound **19c** (225 mg, 1.2 mmol) and DIPEA (0.65 mL, 3.7 mmol). The crude product was purified by column chromatography eluting with a gradient of DCM/NH₃ in MeOH (7N) (100:0 → 95:5) to yield the product as a pale-yellow solid (237 mg, 47%).

R_f 0.71 (DCM/NH₃ in MeOH (7N), 95:5). **¹H NMR** (400 MHz, CDCl₃) δ 7.16 (s, 1H, **5**), 6.80 (s, 1H, **8**), 5.36 (s, 1H, **NH**). 4.34 (s, 1H, **A**), 4.04 (s, 3H, **OCH₃**), 4.00 (s, 3H, **OCH₃**), 3.06 (s, 2H, **C** eq), 2.59 (s, 2H, **C** ax), 2.22 (d, *J* = 15.2, 1H, **D**), 2.00 – 1.94 (m, 2H, **B** eq), 1.90 – 1.82 (m, 6H, **B** ax, **E**), 1.29 (d, *J* = 12.2, 6H, **F**, **G**). **¹³C NMR** (101 MHz, CDCl₃) δ 159.1, 156.2, 155.0, 149.1, 148.1, 107.3, 106.7, 99.7, 64.2, 56.5, 56.3, 48.2, 48.0 (2C), 32.0 (2C), 28.6 (2C), 26.1, 25.9 (2C). **IR** (neat): 3300, 2933. **HRMS** (ESI): *m/z* found [M+H]⁺ 405.2054, C₂₁H₃₀N₄O₂Cl requires 405.2057.

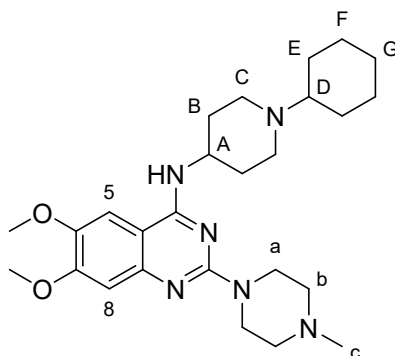
Compound 9: *N*-(1-cyclohexylpiperidin-4-yl)-6,7-dimethoxy-2-(4-methyl-1,4-diazepan-1-yl)-quinazolin-4-amine



Prepared by general procedure **B** with compound **17c** (30 mg, 0.074 mmol) and 1-methylhomopiperazine (0.030 mL, 0.24 mmol). The crude product was purified by column chromatography eluting with a gradient of DCM/NH₃ in MeOH (7N) (100:0 → 90:10) to yield the product as an oily yellow solid (19 mg, 53%) which was ≥95% pure by LC-MS analysis.

R_f 0.39 (DCM/NH₃ in MeOH (7N), 97:3). **m.p.** 79-82 °C. **¹H NMR** (400 MHz, CDCl₃) δ 6.92 (s, 1H, **5**), 6.72 (s, 1H, **8**), 5.02 (d, *J* = 7.2, 1H, **NH**), 4.16 – 4.03 (m, 1H, **A**), 4.02 – 3.99 (m, 2H, **e**), 3.98 (s, 3H, **OCH₃**), 3.96 (s, 3H, **OCH₃**), 3.91 (t, *J* = 6.4, 2H, **a**), 3.12 – 2.90 (m, 2H, **C** eq), 2.82 – 2.67 (m, 2H, **c**), 2.65 – 2.55 (m, 2H, **d**), 2.49 – 2.42 (m, 2H, **C** ax), 2.41 (s, 3H, **f**), 2.21 (d, *J* = 12.5, 2H, **B** eq), 2.13 – 2.00 (m, 2H, **b**), 1.99 – 1.91 (m, 1H, **D**), 1.88 – 1.78 (m, 2H, **B** ax), 1.66 (m, 4H, **E**), 1.39 – 1.19 (m, 6H, **F**, **G**). **¹³C NMR** (101 MHz, CDCl₃) δ 158.5, 158.0, 154.3, 149.4, 145.1, 106.0, 102.8, 100.8, 64.0, 59.0, 57.4, 56.4, 56.0, 48.6, 48.2 (2C), 46.7, 45.9, 45.8, 32.4, 28.8 (2C), 27.8 (2C), 26.3, 26.0 (2C). **IR** (neat): 3313, 2929. **HRMS** (ESI): *m/z* found [M+H]⁺ 483.3437, C₂₇H₄₃N₆O₂ requires 483.3448.

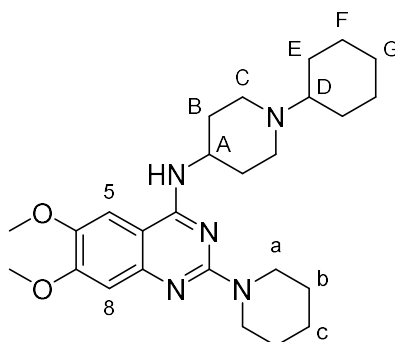
Compound 10: *N*-(1-cyclohexylpiperidin-4-yl)-6,7-dimethoxy-2-(4-methylpiperazin-1-yl)-quinazolin-4-amine



Prepared by general procedure **B** with compound **17c** (30 mg, 0.074 mmol) and 1-methylpiperazine (0.025 mL, 0.23 mmol). The crude product was purified by column chromatography eluting with a gradient of DCM/NH₃ in MeOH (7N) (99:1 → 95:5) to yield the product as a pale-yellow solid (17 mg, 49%) which was ≥95% pure by LC-MS analysis.

R_f 0.26 (DCM/NH₃ in MeOH (7N), 97:3). **m.p.** 80-83 °C. **¹H NMR** (400 MHz, CDCl₃) δ 6.93 (s, 1H, **5**), 6.71 (s, 1H, **8**), 5.00 (d, *J* = 7.3, 1H, **NH**), 4.19 – 4.06 (m, 1H, **A**), 3.98 (s, 3H, **OCH₃**), 3.97 (s, 3H, **OCH₃**), 3.89 (t, *J* = 5.1, 4H, **a**), 3.07 – 2.92 (m, 2H, **C** eq), 2.52 (t, *J* = 5.1, 4H, **b**), 2.49 – 2.41 (m, 1H, **D**), 2.38 (s, 3H, **c**), 2.27 – 2.15 (m, 2H, **C** ax), 1.99 – 1.88 (m, 2H, **B** eq), 1.88 – 1.77 (m, 4H, **E**), 1.74 – 1.54 (m, 2H, **B** ax), 1.37 – 1.22 (m, 6H, **F**, **G**). **¹³C NMR** (101 MHz, CDCl₃) δ 158.9, 158.2, 154.4, 149.2, 145.5, 106.1, 103.2, 100.6, 63.9, 56.3, 56.0, 55.3 (2C), 48.6, 48.2 (2C), 46.4, 44.0 (2C), 32.7 (2C), 28.9 (2C), 26.4, 26.1 (2C). **IR** (neat): 3326, 2929. **HRMS** (ESI): *m/z* found [M+H]⁺ 469.3293, C₂₆H₄₁N₆O₂ requires 469.3291.

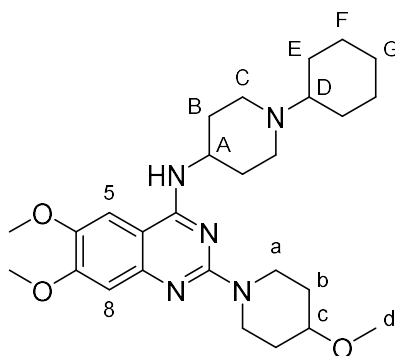
Compound 11: *N*-(1-cyclohexylpiperidin-4-yl)-6,7-dimethoxy-2-(piperidin-1-yl)-quinazolin-4-amine



Prepared by general procedure **B** with compound **17c** (30 mg, 0.074 mmol) and piperidine (0.025 mL, 0.25 mmol). The crude product was purified by column chromatography eluting with a gradient of DCM/NH₃ in MeOH (7N) (99:1 → 92:8) to yield the product as a yellow solid (6 mg, 18%) which was ≥95% pure by LC-MS analysis.

R_f 0.49 (DCM/NH₃ in MeOH (7N), 97.5:2.5). **m.p.** 86-88 °C. **¹H NMR** (400 MHz, CDCl₃) δ 6.92 (s, 1H, **5**), 6.70 (s, 1H, **8**), 4.95 (d, *J* = 7.2, 1H, **NH**), 4.24 – 4.06 (m, 1H, **A**), 3.98 (s, 3H, **OCH₃**), 3.96 (s, 3H, **OCH₃**), 3.83 (dd, *J* = 6.0, 3.9, 4H, **a**), 2.99 (d, *J* = 11.7, 2H, **C** eq), 2.51 – 2.42 (m, 2H, **C** ax), 2.42 – 2.33 (m, 1H, **D**), 2.27 – 2.17 (m, 2H, **B** eq), 1.97 – 1.89 (m, 4H, **E**), 1.84 (d, *J* = 7.2, 2H, **B** ax), 1.71 – 1.56 (m, 6H, **b**, **c**), 1.36 – 1.22 (m, 6H, **F**, **G**). **¹³C NMR** (101 MHz, CDCl₃) δ 159.0, 158.2, 154.3, 149.5, 145.2, 106.1, 102.9, 100.6, 63.9, 56.4, 56.0, 48.5, 48.2 (2C), 45.0 (2C), 32.7 (2C), 28.9 (2C), 26.4, 26.1 (2C), 26.0 (2C), 25.1. **IR** (neat): 3397, 2928. **HRMS** (ESI): *m/z* found [M+H]⁺ 454.3177, C₂₆H₄₀N₅O₃ requires 454.3182.

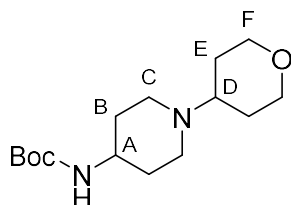
Compound 12: *N*-(1-cyclohexylpiperidin-4-yl)-6,7-dimethoxy-2-(4-methoxypiperidin-1-yl)-quinazolin-4-amine



Prepared by general procedure **C** with compound **17c** (30 mg, 0.074 mmol) and 4-methoxypiperidine (26 mg, 0.23 mmol). The crude product was purified by column chromatography eluting with a gradient of DCM/NH₃ in MeOH (7N) (100:0 → 97.5:2.5) to yield the product as an off-white solid (28 mg, 78%) which was ≥95% pure by LC-MS analysis.

R_f 0.58 (DCM/NH₃ in MeOH (7N), 98:2). **m.p.** 207-210 °C. **¹H NMR** (400 MHz, CDCl₃) δ 6.90 (s, 1H, **5**), 6.72 (s, 1H, **8**), 5.02 (d, *J* = 7.2, 1H, **NH**), 4.46 (dt, *J* = 13.2, 4.6, 2H, **a** eq), 4.16 – 4.07 (m, 1H, **A**), 3.96 (s, 3H, **OCH₃**), 3.94 (s, 3H, **OCH₃**), 3.47 – 3.42 (m, 1H, **c**), 3.41 (s, 3H **d**), 3.30 (ddd, *J* = 13.2, 10.0, 3.2, 2H, **a** ax), 2.99 – 2.93 (m, 2H, **C** eq), 2.44 (td, *J* = 11.8, 2.6, 2H, **C** ax), 2.39 – 2.31 (m, 1H, **D**), 2.23 – 2.15 (m, 2H, **B** eq), 2.04 – 1.97 (m, 2H, **B** ax), 1.93 – 1.79 (m, 4H, **b**), 1.68 – 1.54 (m, 6H, **E**, **F** eq), 1.31 – 1.22 (m, 4H, **F** ax, **G**). **¹³C NMR** (101 MHz, CDCl₃) δ 158.9, 158.2, 154.3, 149.4, 145.3, 106.1, 103.0, 100.7, 77.2, 63.8, 56.3, 56.0, 55.6, 48.6, 48.2 (2C), 41.8 (2C), 32.8 (2C), 30.9 (2C), 29.0 (2C), 26.4, 26.1 (2C). **IR** (neat): 3270, 2927. **HRMS** (ESI): *m/z* found [M+H]⁺ 484.3293, C₂₇H₄₂N₅O₃ requires 484.3288.

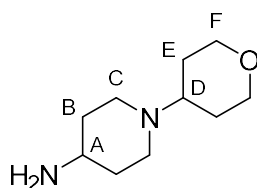
Compound 18d: *tert*-butyl 1-(tetrahydro-2*H*-pyran-4-yl)-piperidin-4-ylcarbamate



Prepared by general procedure **D** with 4-(*N*-Boc-amino)-piperidine (901 mg, 4.5 mmol), NaBH(OAc)₃ (890 mg, 4.2 mmol), and tetrahydro-4*H*-pyran-4-one (0.28 mL, 3.0 mmol), with the addition of acetic acid (0.20 mL). The crude product was purified by column chromatography eluting with DCM/NH₃ in MeOH (7N) (97:3) to yield the product as a white solid (696 mg, 82%).

*R*_f 0.60 (DCM/NH₃ in MeOH (7N), 97:3). ¹H NMR (400 MHz, CDCl₃) δ 4.52 – 4.38 (m, 1H, **A**), 4.04 (dd, *J* = 11.4, 4.4, 2H, **F** eq), 3.47 – 3.31 (m, 2H, **F** ax), 2.92 (d, *J* = 11.4, 2H, **C** eq), 2.55– 2.38 (m, 3H, **C** ax, **D**), 2.32 – 2.18 (m, 2H, **E** eq), 1.98 (d, *J* = 12.4, 2H, **B** eq), 1.76 (d, *J* = 12.4, 2H, **B** ax), 1.67 – 1.55 (m, 2H, **E** ax), 1.47 (s, 9H, Boc (CH₃)₃). HRMS (ESI): *m/z* found [M+H]⁺ 285.2170, C₁₅H₂₉N₂O₃ requires 285.2178.

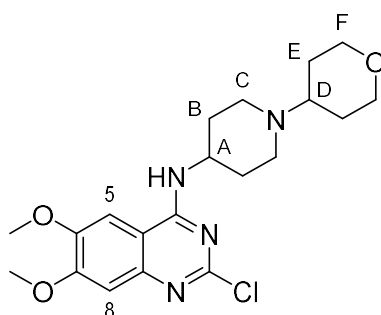
Compound 19d: 1-(tetrahydro-2*H*-pyran-4-yl)-piperidin-4-amine



Prepared by general procedure **E** with *Tert*-butyl compound **18d** (680 mg, 2.4 mmol), the product was collected as a brown oil (225 mg, 51%).

¹H NMR (400 MHz, CDCl₃) δ 4.13 – 3.96 (m, 2H, **F** eq), 3.39 (td, *J* = 11.9, 2.0, 2H, **F** ax), 2.97 (d, *J* = 11.8, 2H, **C** eq), 2.80 – 2.62 (m, 1H, **A**), 2.63 – 2.46 (s, 1H, **D**), 2.37 – 2.17 (m, 2H, **C** ax), 1.89 (d, *J* = 12.8, 2H, **B** eq), 1.83 – 1.74 (m, 2H, **E** eq), 1.69 – 1.58 (m, 2H, **B** ax), 1.51 – 1.34 (m, 2H, **E** ax).

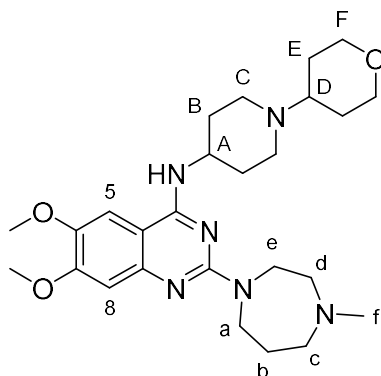
Compound 17d: 2-chloro-6,7-dimethoxy-*N*-(1-(tetrahydro-2*H*-pyran-4-yl)-piperidin-4-yl)-quinazolin-4-amine



Prepared by general procedure **A** with 6,7-dimethoxy-2,4-dichloroquinazoline (311 mg, 1.2 mmol), compound **19d** (220 mg, 1.2 mmol) and DIPEA (0.65 mL, 3.7 mmol). The crude product was purified by column chromatography eluting with a gradient of DCM/NH₃ in MeOH (7N) (99:1 → 95:5) to yield the product as a pale-yellow solid (119 mg, 24%).

R_f 0.27 (DCM/NH₃ in MeOH (7N), 97:3). **¹H NMR** (400 MHz, CDCl₃) δ 7.17 (s, 1H, **5**), 6.79 (s, 1H, **8**), 4.41 – 4.27 (m, 1H, **A**), 4.12 – 4.05 (m, 2H, **F** eq), 4.04 (s, 3H, OCH₃), 4.01 (s, 3H, OCH₃), 3.43 (t, *J* = 11.7, 2H, **F** ax), 3.17 – 3.06 (m, 2H, **C** eq), 2.68 – 2.60 (m, 1H, **D**), 2.60 – 2.44 (m, 2H, **C** ax), 2.25 (d, *J* = 12.7, 2H, **B** eq), 1.92 – 1.77 (m, 2H, **B** ax), 1.77 – 1.64 (m, 4H, **E**). **HRMS** (ESI): *m/z* found [M+H]⁺ 407.1853, C₂₀H₂₈N₄O₃Cl requires 407.1850.

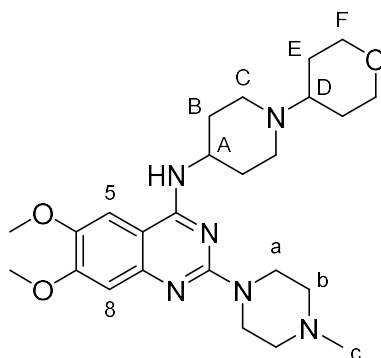
Compound 13: 6,7-dimethoxy-2-(4-methyl-1,4-diazepan-1-yl)-*N*-(1-(tetrahydro-2*H*-pyran-4-yl)-piperidin-4-yl)-quinazolin-4-amine



Prepared by general procedure **B** with compound **17d** (30 mg, 0.074 mmol) and 1-methylhomopiperazine (0.030 mL, 0.24 mmol). The crude product was purified by column chromatography eluting with a gradient of DCM/NH₃ in MeOH (7N) (99:1 → 92:8) to yield the product as a pale-yellow solid (10 mg, 28%) which was ≥95% pure by LC-MS analysis.

R_f 0.33 (DCM/NH₃ in MeOH (7N), 97.5:2.5). **m.p.** 88-91 °C. **¹H NMR** (400 MHz, CDCl₃) δ 6.92 (s, 1H, **5**), 6.71 (s, 1H, **8**), 4.96 (d, *J* = 7.0, 1H, **NH**), 4.14 – 4.11 (m, 1H, **A**), 4.10 – 4.05 (m, 2H, **F** eq), 4.03 – 4.00 (m, 2H, **e**), 3.99 (s, 3H, **OCH₃**), 3.96 (s, 3H, **OCH₃**), 3.91 (t, *J* = 6.4, 2H, **a**), 3.42 (td, *J* = 12.0, 1.9, 2H, **F** ax), 3.07 – 2.99 (m, 2H, **C** eq), 2.75 – 2.73 (m, 2H, **c**), 2.64 – 2.58 (m, 2H, **d**), 2.57 – 2.51 (m, 1H, **D**), 2.41 (s, 3H, **f**), 2.38 – 2.33 (m, 2H, **C** ax), 2.23 (d, *J* = 12.2, 2H, **B** eq), 2.08 – 2.02 (m, 2H, **b**), 1.82 (d, *J* = 12.2, 2H, **B** eq), 1.71 – 1.57 (m, 4H, **E**). **¹³C NMR** (101 MHz, CDCl₃) δ 158.5, 158.0, 154.4, 149.5, 145.1, 106.0, 102.7, 100.7, 67.6 (2C), 61.1, 58.9, 57.4, 56.4, 56.0, 48.7, 48.3 (2C), 46.7, 45.9, 45.7, 32.6 (2C), 29.6 (2C), 27.8. **IR** (neat): 3325, 2942. **HRMS** (ESI): *m/z* found [M+H]⁺ 485.3251, C₂₆H₄₁N₆O₃ requires 485.3240.

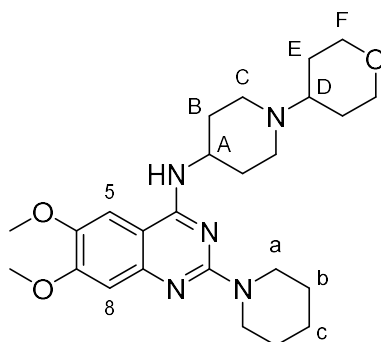
Compound 14: 6,7-dimethoxy-2-(4-methylpiperazin-1-yl)-*N*-(1-(tetrahydro-2*H*-pyran-4-yl)-piperidin-4-yl)-quinazolin-4-amine



Prepared by general procedure **B** with compound **17d** (30 mg, 0.074 mmol) and 1-methylpiperazine (0.025 mL, 0.23 mmol). The crude product was purified by column chromatography eluting with a gradient of DCM/NH₃ in MeOH (7N) (99:1 → 93:7) to yield the product as a pale-yellow solid (16 mg, 45%) which was ≥95% pure by LC-MS analysis.

R_f 0.32 (DCM/NH₃ in MeOH (7N), 98:2). **m.p.** 103-105 °C. **¹H NMR** (400 MHz, CDCl₃) δ 6.93 (s, 1H, **5**), 6.71 (s, 1H, **8**), 5.00 (d, *J* = 7.2, 1H, **NH**), 4.19 – 4.11 (m, 1H, **A**), 4.10 – 4.04 (m, 2H, **F** eq), 3.98 (s, 3H, **OCH₃**), 3.96 (s, 3H, **OCH₃**), 3.89 (d, *J* = 5.7, 4H, **a**), 3.42 (td, *J* = 11.9, 2.0, 2H, **F** ax), 3.03 (d, *J* = 11.7, 2H, **C** eq), 2.59-2.54 (m, 1H, **D**), 2.52 (t, *J* = 5.0, 4H, **b**), 2.41 (dd, *J* = 11.7, 2.5, 2H, **C** eq), 2.37 (s, 3H, **c**), 2.27 – 2.18 (m, 2H, **B** eq), 1.81 (d, *J* = 12.0, 2H, **B** ax), 1.74 – 1.56 (m, 4H, **E**). **¹³C NMR** (101 MHz, CDCl₃) δ 158.9, 158.2, 154.4, 149.2, 145.5, 106.2, 103.1, 100.5, 67.6 (2C), 61.1, 56.4, 56.0, 55.3 (2C), 48.5, 48.3 (2C), 46.4, 44.0 (2C), 32.6 (2C), 29.6 (2C). **IR** (neat): 3332, 2942. **HRMS** (ESI): *m/z* found [M+H]⁺ 471.3083, C₂₅H₃₉N₆O₃ requires 471.3084.

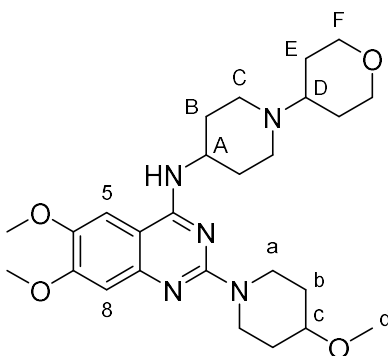
Compound 15: 6,7-dimethoxy-2-(piperidin-1-yl)-*N*-(1-(tetrahydro-2*H*-pyran-4-yl)-piperidin-4-yl)-quinazolin-4-amine



Prepared by general procedure **B** with compound **17d** (30 mg, 0.074 mmol) and piperidine (0.025 mL, 0.25 mmol). The crude product was purified by column chromatography eluting with a gradient of DCM/NH₃ in MeOH (7N) (99:1 → 93:7) to yield the product as a yellow solid (18 mg, 53%) which was ≥95% pure by LC-MS analysis.

R_f 0.44 (DCM/NH₃ in MeOH (7N), 97.5:2.5). **m.p.** 90-93 °C. **¹H NMR** (400 MHz, CDCl₃) δ 6.91 (s, 1H, **5**), 6.73 (s, 1H, **8**), 5.03 (d, *J* = 7.2, 1H, **NH**), 4.18 – 4.10 (m, 1H, **A**), 4.09 – 4.02 (m, 2H, **F** eq), 3.96 (s, 3H, **OCH₃**), 3.93 (s, 3H, **OCH₃**), 3.82 (t, *J* = 4.9, 4H, **a**), 3.40 (td, *J* = 12.0, 2.1, 2H, **F** ax), 3.01 (dt, *J* = 11.9, 3.6, 2H, **C** eq), 2.53 (tt, *J* = 11.5, 3.8, 1H, **D**), 2.38 (td, *J* = 11.9, 2.6, 2H, **C** ax), 2.26 – 2.16 (m, 2H, **B** eq), 1.84 – 1.74 (m, 2H, **B** ax), 1.71 – 1.54 (m, 10H, **b**, **c**, **E**). **¹³C NMR** (101 MHz, CDCl₃) δ 159.0, 158.2, 154.3, 149.5, 145.2, 106.1, 102.9, 100.7, 67.6 (2C), 61.1, 56.3, 56.0, 48.5, 48.3 (2C), 45.1 (2C), 32.6 (2C), 29.6 (2C), 26.0 (2C), 25.1. **IR** (neat): 3389, 2928. **HRMS** (ESI): *m/z* found [M+H]⁺ 456.2970, C₂₅H₃₈N₅O₃ requires 456.2975.

Compound 16: 6,7-dimethoxy-2-(4-methoxypiperidin-1-yl)-*N*-(1-(tetrahydro-2-pyran-4-yl)-piperidin-4-yl)-quinazolin-4-amine

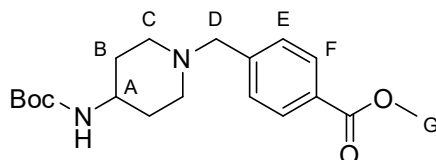


Prepared by general procedure **B** with compound **17d** (30 mg, 0.074 mmol) and 4-methoxypiperidine (26 mg, 0.23 mmol). The crude product was purified by column chromatography eluting with a gradient of DCM/NH₃ in MeOH (7N) (99:1 → 93:7) to yield the product as a pale-yellow solid (27 mg, 75%) which was ≥95% pure by LC-MS analysis.

R_f 0.40 (DCM/NH₃ in MeOH (7N), 97.5:2.5). **m.p.** 103-107 °C. **¹H NMR** (400 MHz, CDCl₃) δ 6.91 (s, 1H, **5**), 6.73 (s, 1H, **8**), 5.09 – 4.99 (m, 1H, **NH**), 4.54 – 4.39 (m, 2H, **a** eq), 4.17 – 4.09 (m, 1H, **A**), 4.08 – 4.02 (m, 2H, **F** eq), 3.96 (s, 3H, **OCH₃**), 3.94 (s, 3H, **OCH₃**), 3.49 – 3.42 (m, 1H, **c**), 3.41 (s, 3H, **d**), 3.30 (ddd, *J* = 13.3, 9.9, 3.2, 2H, **F** ax), 3.04 – 2.96 (m, 2H, **C** eq), 2.53 (tt, *J* = 11.3, 3.8, 1H, **D**), 2.38 (td, *J* = 11.9, 2.6, 2H, **C** ax), 2.25 – 2.17 (m, 2H, **B** eq), 2.04 – 1.96 (m, 2H, **b** eq), 1.84 – 1.76 (m, 2H, **B** ax), 1.71 – 1.52 (m, 6H, **b** ax, **E**). **¹³C NMR** (101 MHz, CDCl₃) δ 158.8, 158.2, 154.4, 149.4, 145.4, 106.1, 102.9, 100.7, 77.2, 67.6 (2C), 61.1, 56.3, 56.0, 55.6, 48.5, 48.3 (2C), 41.8 (2C), 32.6 (2C), 30.9 (2C), 29.6 (2C). **IR** (neat): 3348, 2935. **HRMS** (ESI): *m/z* found [M+H]⁺ 486.3082, C₂₆H₄₀N₅O₄ requires 486.3080.

4.1.3. Expanding the SAR: Investigating Aromatic Substituents

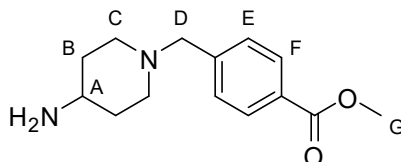
Compound 18e: methyl 4-((4-(*tert*-butoxycarbonylamino)-piperidin-1-yl)-methyl)-benzoate



Prepared by general procedure **D** with 4-(*N*-Boc-amino)-piperidine (901 mg, 4.5 mmol), NaBH(OAc)₃ (890 mg, 4.2 mmol) and methyl 4-formylbenzoate (492 mg, 3.0 mmol). The crude product was purified by column chromatography eluting with EtOAc/pentane (75:25) to yield the product as a white solid (796 mg, 76%).

*R*_f 0.31 (EtOAc/pentane, 70:30). **¹H NMR** (400 MHz, CDCl₃) δ 8.01 (d, *J* = 8.2, 2H, **F**), 7.44 – 7.38 (m, 2H, **E**), 4.45 (s, 1H, **A**), 3.94 (s, 3H, **G**), 3.55 (s, 2H, **D**), 2.80 (d, *J* = 11.4, 2H, **C** eq), 2.13 (t, *J* = 11.4, 2H, **C** ax), 1.94 (d, *J* = 12.6, 2H, **B** eq), 1.47 (s, 9H, Boc (CH₃)₃), 1.49 – 1.38 (m, 2H, **B** ax). **HRMS** (ESI): *m/z* found [M+H]⁺ 349.2121, C₁₉H₂₉N₂O₄ requires 349.2127.

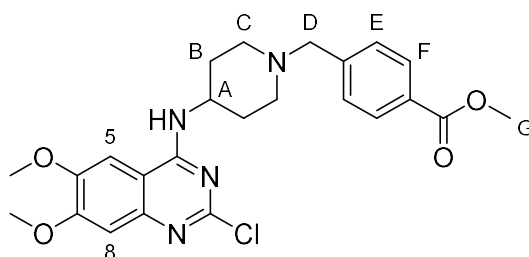
Compound 19e: methyl 4-((4-aminopiperidin-1-yl)-methyl)-benzoate



Prepared by general procedure **E** with compound **18e** (780 mg, 2.2 mmol), the product was collected as a brown oil (430 mg, 77%).

¹H NMR (400 MHz, CDCl₃) δ 8.03 – 7.98 (m, 2H, **F**), 7.44 – 7.39 (m, 2H, **E**), 3.93 (s, 3H, **G**), 3.57 (s, 2H, **D**), 2.83 (d, *J* = 11.8, 2H, **C** eq), 2.75 – 2.64 (m, 1H, **A**), 2.08 (t, *J* = 11.8, 2H, **C** ax), 1.82 (d, *J* = 12.7, 2H, **B** eq), 1.48 – 1.36 (m, 2H, **B** ax). **HRMS** (ESI): *m/z* found [M+H]⁺ 249.1604, C₁₄H₂₁N₂O₂ requires 249.1603.

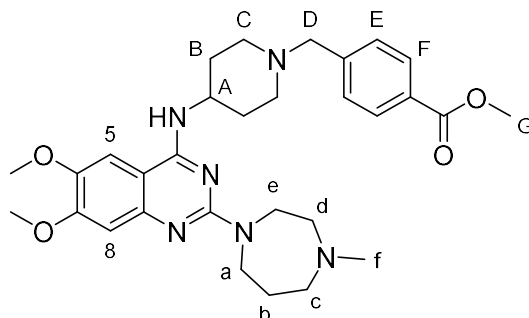
Compound 17e: methyl 4-((4-(2-chloro-6,7-dimethoxyquinazolin-4-ylamino)-piperidin-1-yl)-methyl)-benzoate



Prepared by general procedure **A** with 6,7-dimethoxy-2,4-dichloroquinazoline (438 mg, 1.7 mmol), compound **19e** (420 mg, 1.7 mmol) and DIPEA (0.90 mL, 5.2 mmol). The crude product was purified by column chromatography eluting with a gradient of DCM/NH₃ in MeOH (7N) (100:0 → 95:5) to yield the product as a pale-yellow solid (396 mg, 50%).

¹H NMR (400 MHz, CDCl₃) δ 8.03 (d, *J* = 8.1 2H, **F**), 7.45 (d, *J* = 8.1, 2H, **E**), 7.16 (s, 1H, **5**), 6.77 (s, 1H, **8**), 5.28 (d, *J* = 7.7, 1H, **NH**), 4.36 – 4.26 (m, 1H, **A**), 4.03 (s, 3H, **OCH₃**), 4.00 (s, 3H, **OCH₃**), 3.94 (s, 3H, **G**), 3.63 (s, 2H, **D**), 2.91 (d, *J* = 11.4, 2H, **C** eq), 2.30 (t, *J* = 11.4, 2H, **C** ax), 2.18 (d, *J* = 12.4, 2H, **B** eq), 1.72 – 1.60 (m, 2H, **B** ax). **¹³C NMR** (101 MHz, CDCl₃) δ 167.1, 159.1, 156.2, 155.0, 149.0, 148.1, 144.0, 129.6 (2C), 129.0, 128.8 (2C), 107.4, 106.6, 99.4, 62.6, 56.4, 56.3, 52.4 (2C), 52.1, 48.3, 32.3 (2C). **IR** (neat): 3302, 2934, 1719. **HRMS** (ESI): *m/z* found [M+H]⁺ 471.1814, C₂₄H₂₈N₄O₄Cl requires 471.1799.

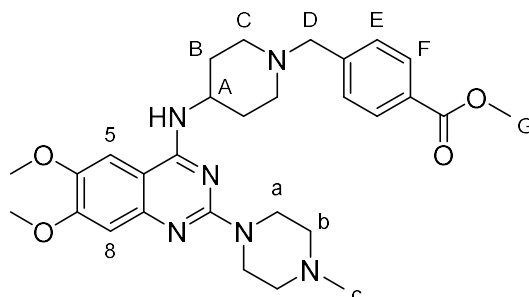
Compound 20: methyl 4-((4-(6,7-dimethoxy-2-(4-methyl-1,4-diazepan-1-yl)-quinazolin-4-ylamino)-piperidin-1-yl)-methyl)-benzoate



Prepared by general procedure **B** with compound **17e** (30 mg, 0.064 mmol) and 1-methylhomopiperazine (0.025 mL, 0.20 mmol). The crude product was purified by column chromatography eluting with DCM/NH₃ in MeOH (7N) (99:1) to yield the product as an off-white solid (15 mg, 43%) which was ≥95% pure by LC-MS analysis.

R_f 0.41 (DCM/NH₃ in MeOH (7N), 98:2). **m.p.** 79-83 °C. **¹H NMR** (400 MHz, CDCl₃) δ 8.02 (d, *J* = 8.2, 2H, **F**), 7.45 (d, *J* = 8.2, 2H, **E**), 6.91 (s, 1H, **5**), 6.72 (s, 1H, **8**), 5.00 (d, *J* = 7.0, 1H, **NH**), 4.18 – 4.08 (m, 1H, **A**), 4.01 – 3.99 (m, 2H, **e**), 3.97 (s, 3H, **OCH₃**), 3.95 (s, 3H, **OCH₃**), 3.93 (s, 3H, **G**), 3.90 (t, *J* = 6.3, 2H, **a**), 3.61 (s, 2H, **D**), 2.91 (d, *J* = 11.4, 2H, **C** eq), 2.75 – 2.70 (m, 2H, **c**), 2.63 – 2.55 (m, 2H, **d**), 2.39 (s, 3H, **f**), 2.27 – 2.22 (m, 2H, **C** ax), 2.21 – 2.14 (m, 2H, **B** eq), 2.04 (p, *J* = 6.3, 2H, **b**), 1.72 – 1.59 (m, 2H, **B** ax). **¹³C NMR** (101 MHz, CDCl₃) δ 167.1, 158.6, 158.0, 154.4, 149.6, 145.0, 144.2, 129.6 (2C), 129.0, 128.8 (2C), 106.1, 102.7, 100.7, 62.7, 59.0, 57.4, 56.4, 56.0, 52.7 (2C), 52.1, 48.3, 46.8, 45.9, 45.8, 32.3 (2C), 27.9. **IR** (neat): 3327, 2941, 1717. **HRMS** (ESI): *m/z* found [M+H]⁺ 549.3209, C₃₀H₄₁N₆O₄ requires 549.3189.

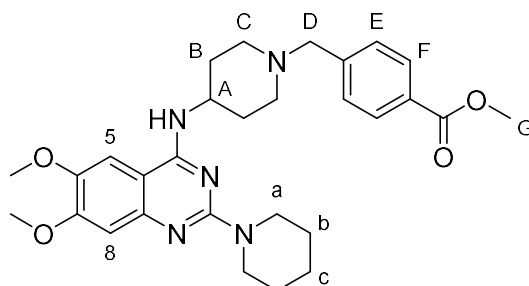
Compound 21: methyl 4-((4-(6,7-dimethoxy-2-(4-methylpiperazin-1-yl)-quinazolin-4-ylamino)-piperidin-1-yl)-methyl)-benzoate



Prepared by general procedure **B** with compound **17e** (30 mg, 0.064 mmol) and 1-methylpiperazine (0.025 mL, 0.23 mmol). The crude product was purified by column chromatography eluting with a gradient of DCM/NH₃ in MeOH (7N) (100:0 → 95:5) to yield the product as an off-white solid (17 mg, 50%) which was ≥95% pure by LC-MS analysis.

R_f 0.46 (DCM/NH₃ in MeOH (7N), 98:2). **m.p.** 79-82 °C. **¹H NMR** (400 MHz, CDCl₃) δ 8.02 (d, *J* = 8.2, 1H), 7.44 (d, *J* = 8.2, 2H), 6.93 (s, 1H, **5**), 6.72 (s, 1H, **8**), 5.04 (d, *J* = 7.1, 1H, **NH**), 4.21 – 4.10 (m, 1H, **A**), 3.97 (s, 3H, **OCH₃**), 3.95 (s, 3H, **OCH₃**), 3.93 (s, 3H, **G**), 3.88 (dd, *J* = 6.5, 3.9, 4H, **a**), 3.61 (s, 2H, **D**), 2.91 (d, *J* = 11.5, 2H, **C** eq), 2.51 (t, *J* = 5.0, 4H, **b**), 2.36 (s, 3H, **c**), 2.25 (td, *J* = 11.5, 2.5, 2H, **C** ax), 2.19 (s, 2H, **B** eq), 1.72 – 1.58 (m, 2H, **B** ax). **¹³C NMR** (101 MHz, CDCl₃) δ 167.1, 158.9, 158.2, 154.4, 149.2, 145.5, 144.1, 129.6 (2C), 129.0, 128.8 (2C), 106.2, 103.1, 100.6, 62.7, 56.3, 56.0, 55.3 (2C), 52.7 (2C), 52.1, 48.2, 46.3, 44.0 (2C), 32.3 (2C). **IR** (neat): 3397, 2940, 1718. **HRMS** (ESI): *m/z* found [M+H]⁺ 535.3051, C₂₉H₃₉N₆O₄ requires 535.3051.

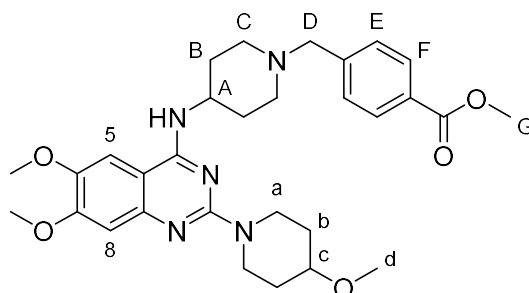
Compound 22: methyl 4-((4-(6,7-dimethoxy-2-(piperidin-1-yl)-quinazolin-4-ylamino)-piperidin-1-yl)-methyl)-benzoate



Prepared by general procedure **B** with compound **17e** (30 mg, 0.064 mmol) and piperidine (0.020 mL, 0.20 mmol). The crude product was purified by column chromatography eluting with a gradient of DCM/NH₃ in MeOH (7N) (100:0 → 97:3) to yield the product as an off-white solid (30 mg, 90%) which was ≥95% pure by LC-MS analysis.

R_f 0.71 (DCM/NH₃ in MeOH (7N), 97.5:2.5). **m.p.** 81-84 °C. **¹H NMR** (400 MHz, CDCl₃) δ 8.02 (d, *J* = 8.2, 2H, **F**), 7.44 (d, *J* = 8.2, 2H, **E**), 6.92 (s, 1H, **5**), 6.73 (s, 1H, **8**), 5.03 (s, 1H, **NH**), 4.23 – 4.10 (m, 1H, **A**), 3.96 (s, 3H, **OCH₃**), 3.94 (s, 3H, **OCH₃**), 3.93 (s, 3H, **G**), 3.85 – 3.79 (m, 4H, **a**), 3.61 (s, 2H, **D**), 2.95 – 2.84 (m, 2H, **C** eq), 2.25 (td, *J* = 11.4, 2.6, 2H, **C** ax), 2.20 – 2.12 (m, 2H, **B** eq), 1.71 – 1.57 (m, 8H, **B** eq, **b**, **c**). **¹³C NMR** (101 MHz, CDCl₃) δ 167.1, 159.0, 158.2, 154.3, 149.5, 145.2, 144.2, 129.6 (2C), 129.0, 128.8 (2C), 106.1, 102.8, 100.7, 62.7, 56.3, 56.0, 52.7 (2C), 52.1, 48.2, 45.1 (2C), 32.3 (2C), 26.0 (2C), 25.1. **IR** (neat): 3399, 2931, 1720. **HRMS** (ESI): *m/z* found [M+H]⁺ 520.2913, C₂₉H₃₈N₅O₄ requires 520.2924.

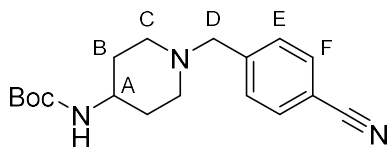
Compound 23: methyl 4-((4-(6,7-dimethoxy-2-(4-methoxypiperidin-1-yl)-quinazolin-4-ylamino)-piperidin-1-yl)-methyl)-benzoate



Prepared by general procedure **B** with compound **17e** (30 mg, 0.064 mmol) and 4-methoxypiperidine (25 mg, 0.22 mmol). The crude product was purified by column chromatography eluting with a gradient of DCM/NH₃ in MeOH (7N) (100:0 → 96:4) to yield the product as a pale pink solid (10 mg, 28%) which was ≥95% pure by LC-MS analysis.

R_f 0.48 (DCM/NH₃ in MeOH (7N), 98:2). **m.p.** 99-101 °C. **¹H NMR** (400 MHz, CDCl₃) δ 8.02 (d, *J* = 8.2, 2H, **F**), 7.44 (d, *J* = 8.2, 2H, **E**), 6.91 (s, 1H, **5**), 6.72 (s, 1H, **8**), 5.02 (d, *J* = 7.0, 1H, **NH**), 4.45 (dt, *J* = 13.4, 4.6, 2H, **a** eq), 4.22 – 4.10 (m, 1H, **A**), 3.97 (s, 3H, **OCH₃**), 3.95 (s, 3H, **OCH₃**), 3.93 (s, 3H, **G**), 3.61 (s, 2H, **D**), 3.49 – 3.44 (m, 1H, **c**), 3.41 (s, 3H, **d**), 3.37 – 3.25 (m, 2H, **a** ax), 2.97 – 2.85 (m, 2H, **C** eq), 2.25 (td, *J* = 11.4, 2.5, 2H, **C** ax), 2.20 – 2.15 (m, 2H, **B** eq), 2.05 – 1.95 (m, 2H, **B** ax), 1.70 – 1.54 (m, 4H, **b**). **¹³C NMR** (101 MHz, CDCl₃) δ 167.1, 158.8, 158.2, 154.4, 149.4, 145.4, 144.2, 129.6 (2C), 129.0, 128.8 (2C), 106.1, 102.9, 100.6, 62.7, 56.3, 56.0, 55.6, 53.4, 52.7 (2C), 52.1, 48.2, 41.8 (2C), 32.3 (2C), 30.9 (2C). **IR** (neat): 3398, 2938, 1719. **HRMS** (ESI): *m/z* found [M+H]⁺ 550.3032, C₃₀H₄₀N₅O₅ requires 550.3029.

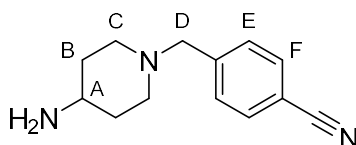
Compound 18f: *tert*-butyl 1-(4-cyanobenzyl)-piperidin-4-ylcarbamate



Prepared by general procedure **D** with 4-(*N*-Boc-amino)-piperidine (1.50 g, 7.5 mmol), NaBH(OAc)₃ (1.48 g, 7.0 mmol) and 4-formylbenzonitrile (656 mg, 5.0 mmol). The crude product was purified by column chromatography eluting with a gradient of DCM/NH₃ in MeOH (7N) (100:0 → 95:5) to yield the product as a white solid (1.17 g, 74%).

*R*_f 0.20 (EtOAc/pentane, 50:50). **¹H NMR** (400 MHz, CDCl₃) δ 7.66 – 7.59 (m, 2H, **F**), 7.48 – 7.42 (m, 2H, **E**), 4.50 – 4.38 (m, 1H, **A**), 3.54 (s, 2H, **D**), 2.77 (d, *J* = 11.6, 2H, **C** eq), 2.14 (td, *J* = 11.6, 2.6, 2H, **C** ax), 1.94 (d, *J* = 12.7, 2H, **B** eq), 1.59 – 1.56 (m, 2H, **B** ax), 1.47 (s, 9H, Boc (CH₃)₃). **HRMS** (ESI): *m/z* found [M+H]⁺ 316.2027, C₁₈H₂₆N₃O₂ requires 316.2025.

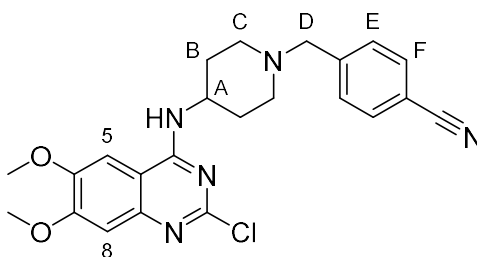
Compound 19f: 4-((4-aminopiperidin-1-yl)-methyl)-benzonitrile



Prepared by general procedure **E** with compound **18f** (780 mg, 2.2 mmol), the product was collected as a brown oil (682 mg, 88%).

¹H NMR (400 MHz, CDCl₃) δ 7.62 (d, *J* = 8.5, 1H, **F**), 7.46 (d, *J* = 8.5, 2H, **E**), 3.54 (s, 2H, **D**), 2.80 (ddt, *J* = 12.0, 4.8, 2.4, 2H, **C** eq), 2.75 – 2.66 (m, 1H, **A**), 2.07 (td, *J* = 12.0, 2.5, 2H, **C** ax), 1.86 – 1.77 (m, 2H, **B** eq), 1.41 (ddt, *J* = 11.7, 4.1, 1.0, 2H, **B** ax).

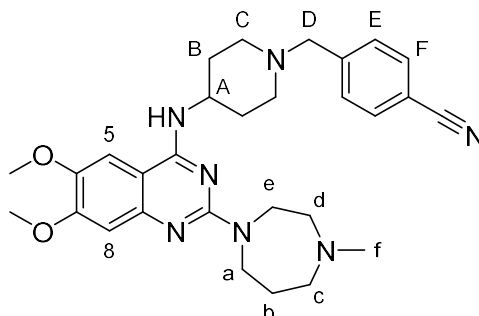
Compound 17f: 4-((4-(2-chloro-6,7-dimethoxyquinazolin-4-ylamino-)-piperidin-1-yl)-methyl)-benzonitrile



Prepared by general procedure **A** with 6,7-dimethoxy-2,4-dichloroquinazoline (821 mg, 3.2 mmol), compound **19f** (682 mg, 3.2 mmol) and DIPEA (1.70 mL, 9.8 mmol). The crude product was purified by column chromatography eluting with a gradient of DCM/NH₃ in MeOH (7N) (100:0 → 95:5) to yield the product as a pale-yellow solid (441 mg, 32%).

R_f 0.53 (DCM/NH₃ in MeOH (7N), 98:2). **¹H NMR** (400 MHz, CDCl₃) δ 7.65 (d, *J* = 8.1, 2H, **F**), 7.50 (d, *J* = 8.1, 2H, **E**), 7.17 (s, 1H, **5**), 6.78 (s, 1H, **8**), 5.28 (d, *J* = 7.8, 1H, **NH**), 4.39 – 4.27 (m, 1H, **A**), 4.03 (s, 3H, **OCH₃**), 4.00 (s, 3H, **OCH₃**), 3.62 (s, 2H, **D**), 2.90 (d, *J* = 11.5, 2H, **C** eq), 2.31 (t, *J* = 11.5, 2H, **C** ax), 2.19 (d, *J* = 12.5, 2H, **B** eq), 1.72 – 1.60 (m, 2H, **B** ax). **¹³C NMR** (101 MHz, CDCl₃) δ 159.0, 156.2, 155.0, 149.1, 148.1, 144.5, 132.2 (2C), 129.4 (2C), 129.1, 119.0, 110.9, 107.4, 99.4, 62.4, 56.4, 56.3, 52.5 (2C), 48.2, 32.3 (2C). **IR** (neat): 3393, 2927, 2222. **HRMS** (ESI): *m/z* found [M+H]⁺ 438.1703, C₂₃H₂₅N₅O₂Cl requires 438.1697.

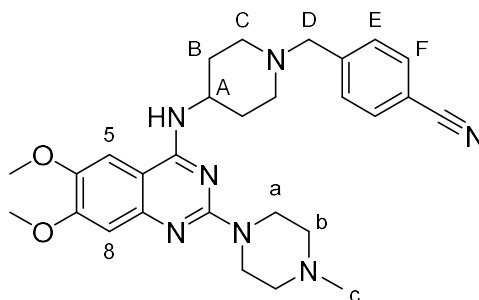
Compound 24: 4-((4-(6,7-dimethoxy-2-(4-methyl-1,4-diazepan-1-yl)-quinazolin-4-ylamino)-piperidin-1-yl)-methyl)-benzonitrile



Prepared by general procedure **B** with compound **17f** (80 mg, 0.18 mmol) and 1-methylhomopiperazine (0.070 mL, 0.56 mmol). The crude product was purified by column chromatography eluting a gradient of DCM/NH₃ in MeOH (7N) (100:0 → 97:3) to yield the product as an off-white solid (58 mg, 61%) which was ≥95% pure by LC-MS analysis.

R_f 0.27 (DCM/NH₃ in MeOH (7N), 98:2). **m.p.** 107–110 °C. **¹H NMR** (400 MHz, CDCl₃) δ 7.63 (d, *J* = 8.0, 2H, **F**), 7.49 (d, *J* = 8.0, 2H, **E**), 6.90 (s, 1H, **5**), 6.74 (s, 1H, **8**), 5.05 (d, *J* = 7.0, 1H, **NH**), 4.18 – 4.09 (m, 1H, **A**), 4.01 – 3.97 (m, 2H, **e**), 3.96 (s, 3H, **OCH₃**), 3.94 (s, 3H, **OCH₃**), 3.89 (t, *J* = 6.4, 2H, **a**), 3.60 (s, 2H, **D**), 2.88 (d, *J* = 11.9, 2H, **C** eq), 2.74 – 2.67 (m, 2H, **c**), 2.61 – 2.55 (m, 2H, **d**), 2.38 (s, 3H, **f**), 2.27 – 2.15 (m, 4H, **C** ax, **b**), 2.07 – 1.97 (m, 2H, **B** eq), 1.70 – 1.59 (m, 2H, **B** ax). **¹³C NMR** (101 MHz, CDCl₃) δ 158.6, 158.0, 154.3, 149.7, 145.0, 144.7, 132.1 (2C), 129.3 (2C), 119.0, 110.8, 106.1, 102.8, 100.7, 62.5, 59.0, 57.4, 56.4, 56.0, 52.8 (2C), 48.3, 46.8, 45.9, 32.3 (2C), 30.9, 27.9. **IR** (neat): 3396, 2935, 2228. **HRMS** (ESI): *m/z* found [M+H]⁺ 516.3090, C₂₉H₃₈N₇O₂ requires 516.3087.

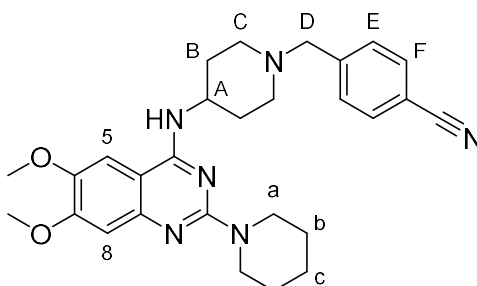
Compound 25: 4-((4-(6,7-dimethoxy-2-(4-methylpiperazin-1-yl)-quinazolin-4-ylamino)-piperidin-1-yl)-methyl)-benzonitrile



Prepared by general procedure **B** with compound **17f** (80 mg, 0.18 mmol) and 1-methylpiperazine (0.060 mL, 0.54 mmol). The crude product was purified by column chromatography eluting a gradient of DCM/NH₃ in MeOH (7N) (100:0 → 97:3) to yield the product as an off-white solid (85 mg, 93%) which was ≥95% pure by LC-MS analysis.

R_f 0.38 (DCM/NH₃ in MeOH (7N), 98:2). **m.p.** 173-176 °C. **¹H NMR** (400 MHz, CDCl₃) δ 7.63 (d, *J* = 8.2, 2H, **F**), 7.47 (d, *J* = 8.2, 2H, **E**), 6.92 (s, 1H, **5**), 6.77 (s, 1H, **8**), 5.16 (d, *J* = 7.2, 1H, **NH**), 4.24 – 4.08 (m, 1H, **A**), 3.95 (s, 3H, **OCH₃**), 3.94 (s, 3H, **OCH₃**), 3.90 – 3.82 (m, 4H, **a**), 3.60 (s, 2H, **D**), 2.88 (dt, *J* = 11.8, 3.5, 2H, **C** eq), 2.50 (t, *J* = 5.1, 4H, **b**), 2.35 (s, 3H, **c**), 2.25 (td, *J* = 11.8, 2.5, 2H, **C** ax), 2.21 – 2.13 (m, 2H, **B** eq), 1.71 – 1.57 (m, 2H, **B** ax). **¹³C NMR** (101 MHz, CDCl₃) δ 158.8, 158.2, 154.4, 149.2, 145.5, 144.7, 132.1 (2C), 129.4 (2C), 119.0, 110.8, 106.1, 103.2, 100.7, 62.5, 56.3, 56.0, 55.3 (2C), 52.8 (2C), 48.1, 46.3, 44.0 (2C), 32.2 (2C). **IR** (neat): 3399, 2933, 2227. **HRMS** (ESI): *m/z* found [M+H]⁺ 502.2910, C₂₈H₃₆N₇O₂ requires 502.2930.

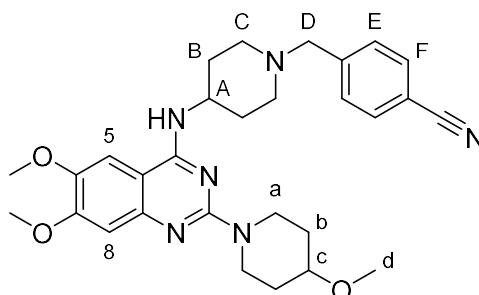
Compound 26: 4-((4-(6,7-dimethoxy-2-(piperidin-1-yl)-quinazolin-4-ylamino)-piperidin-1-yl)-methyl)-benzonitrile



Prepared by general procedure **C** with compound **17f** (80 mg, 0.18 mmol) and piperidine (0.055 mL, 0.56 mmol). The crude product was purified by column chromatography eluting a gradient of DCM/NH₃ in MeOH (7N) (100:0 → 98:2) to yield the product as a pale pinkish brown solid (51 mg, 61%) which was ≥95% pure by LC-MS analysis.

R_f 0.48 (DCM/NH₃ in MeOH (7N), 98:2). **m.p.** 90-92 °C. **¹H NMR** (400 MHz, CDCl₃) δ 7.64 (d, *J* = 8.2, 2H, **F**), 7.49 (d, *J* = 8.2, 2H, **E**), 6.91 (s, 1H, **5**), 6.71 (s, 1H, **8**), 4.96 (d, *J* = 7.1, 1H, **NH**), 4.24 – 4.13 (m, 1H, **A**), 3.98 (s, 3H, OCH₃), 3.96 (s, 3H, OCH₃), 3.82 (t, *J* = 4.9, 4H, **a**), 3.61 (s, 2H, **D**), 2.89 (d, *J* = 11.7, 2H, **C** eq), 2.31 – 2.23 (m, 2H, **C** ax), 2.23 – 2.16 (m, 2H, **B** eq), 1.72 – 1.59 (m, 8H, **B** ax, **b**, **c**). **¹³C NMR** (101 MHz, CDCl₃) δ 159.9, 158.2, 154.4, 149.6, 145.2, 144.7, 132.1 (2C), 129.4 (2C), 119.0, 110.9, 106.2, 102.8, 100.6, 62.5, 56.4, 56.0, 52.8 (2C), 48.1, 45.0 (2C), 32.3 (2C), 26.0 (2C), 25.1. **IR** (neat): 3405, 2927, 2227, 1630. **HRMS** (ESI): *m/z* found [M+H]⁺ 487.2824, C₂₈H₃₅N₆O₂ requires 487.2821.

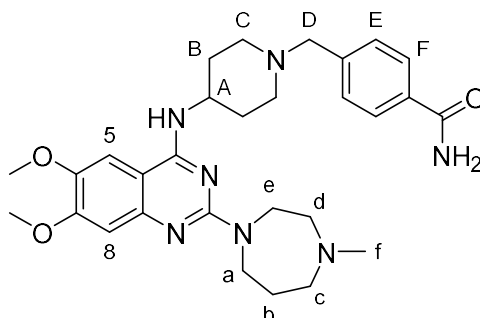
Compound 27: 4-((4-(6,7-dimethoxy-2-(4-methoxypiperidin-1-yl)-quinazolin-4-ylamino)-piperidin-1-yl)-methyl)-benzonitrile



Prepared by general procedure **C** with compound **17f** (75 mg, 0.17 mmol) and 4-methoxypiperidine (60 mg, 0.52 mmol). The crude product was purified by column chromatography eluting a gradient of DCM/NH₃ in MeOH (7N) (100:0 → 98:2) to yield the product as a reddish-brown solid (40 mg, 45%) which was ≥95% pure by LC-MS analysis.

R_f 0.53 (DCM/NH₃ in MeOH (7N), 98:2). **m.p.** 75-78 °C. **¹H NMR** (400 MHz, CDCl₃) δ 7.64 (d, *J* = 8.1, 2H, **F**), 7.49 (d, *J* = 8.1, 2H, **E**), 6.91 (s, 1H, **5**), 6.72 (s, 1H, **8**), 5.01 (d, *J* = 7.1, 1H, **NH**), 4.45 (dt, *J* = 13.3, 4.6, 2H, **a** eq), 4.23 – 4.12 (m, 1H, **A**), 3.97 (s, 3H, **OCH₃**), 3.96 (s, 3H, **OCH₃**), 3.61 (s, 2H, **D**), 3.46 (dt, *J* = 8.3, 4.0, 1H, **c**), 3.41 (s, 3H, **d**), 3.31 (ddt, *J* = 13.3, 9.9, 4.3, 2H, **a** ax), 2.89 (d, *J* = 11.6, 2H, **C** eq), 2.31 – 2.23 (m, 2H, **C** ax), 2.22 – 2.16 (m, 2H, **B** eq), 2.05 – 1.96 (m, 2H, **B** ax), 1.71 – 1.54 (m, 4H, **b**). **¹³C NMR** (101 MHz, CDCl₃) δ 158.8, 158.2, 154.4, 149.4, 145.4, 144.7, 132.1 (2C), 129.4 (2C), 119.0, 110.9, 106.2, 102.9, 100.6, 77.2, 62.5, 56.3, 56.0, 55.6, 52.7 (2C), 48.1, 41.8 (2C), 32.3 (2C), 30.9 (2C). **IR** (neat): 3397, 2934, 2227. **HRMS** (ESI): *m/z* found [M+H]⁺ 517.2939, C₂₉H₃₇N₆O₃ requires 517.2927.

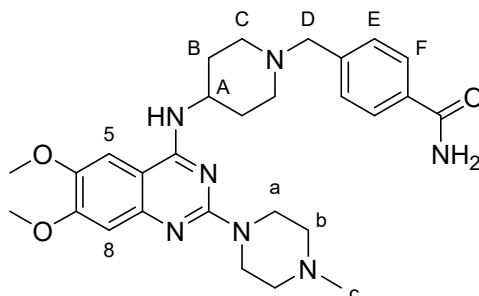
Compound 28: 4-((4-(6,7-dimethoxy-2-(4-methyl-1,4-diazepan-1-yl)-quinazolin-4-ylamino)-piperidin-1-yl)-methyl)-benzamide



Prepared by general procedure **F** with compound **24** (28 mg, 0.054 mmol). The crude product was purified by column chromatography eluting a gradient of DCM/NH₃ in MeOH (7N) (97.5:2.5 → 95:5) to yield the product as a white solid (5 mg, 17%) which was ≥95% pure by LC-MS analysis.

R_f 0.31 (DCM/NH₃ in MeOH (7N), 97:3). **m.p.** 142-144 °C. **¹H NMR** (400 MHz, CDCl₃) δ 7.80 (d, *J* = 8.2, 2H, **F**), 7.47 (d, *J* = 8.2, 2H, **E**), 6.92 (s, 1H, **5**), 6.72 (s, 1H, **8**), 4.99 (s, 1H, **NH**), 4.19 – 4.09 (m, 1H, **A**), 4.02 – 3.99 (m, 2H, **e**), 3.98 (s, 3H, **OCH₃**), 3.96 (s, 3H, **OCH₃**), 3.90 (t, *J* = 6.4, 2H, **a**), 3.62 (s, 2H, **D**), 2.92 (d, *J* = 11.3, 2H, **C** eq), 2.77 – 2.70 (m, 2H, **c**), 2.64 – 2.57 (m, 2H, **d**), 2.40 (s, 3H, **f**), 2.29 – 2.13 (m, 4H, **C** ax, **b**), 2.09 – 2.01 (m, 2H, **B** eq), 1.74 – 1.58 (m, 2H, **B** ax). **¹³C NMR** (101 MHz, CDCl₃) δ 169.0, 158.4, 158.0, 154.4, 149.5, 145.1, 143.3, 132.1, 129.1 (2C), 127.4 (2C), 106.1, 102.7, 100.7, 62.6, 59.0, 57.4, 56.4, 56.0, 52.7 (2C), 48.4, 46.7, 45.9, 45.8, 32.3 (2C), 27.8. **IR** (neat): 3328, 2932, 1662. **HRMS** (ESI): *m/z* found [M+H]⁺ 534.3188, C₂₉H₄₀N₇O₃ requires 534.3193.

Compound 29: 4-((4-(6,7-dimethoxy-2-(4-methylpiperazin-1-yl)-quinazolin-4-ylamino)-piperidin-1-yl)-methyl)-benzamide

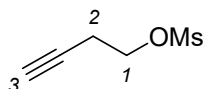


Prepared by general procedure **F** with compound **25** (45 mg, 0.090 mmol). The crude product was purified by column chromatography eluting a gradient of DCM/NH₃ in MeOH (7N) (100:00 → 98:2) to yield the product as a white solid (8 mg, 17%) which was ≥95% pure by LC-MS analysis.

R_f 0.12 (DCM/NH₃ in MeOH (7N), 98:2). **m.p.** 192-196 °C. **¹H NMR** (400 MHz, CDCl₃) δ 7.80 (d, *J* = 8.2, 2H, **F**), 7.47 (d, *J* = 8.2, 2H, **E**), 6.93 (s, 1H, **5**), 6.71 (s, 1H, **8**), 5.00 (d, *J* = 7.1, 1H, **NH**), 4.22 – 4.12 (m, 1H, **A**), 3.98 (s, 3H, OCH₃), 3.97 (s, 3H, OCH₃), 3.92 – 3.85 (m, 4H, **a**), 3.63 (s, 2H, **D**), 2.92 (d, *J* = 11.7, 2H, **C** eq), 2.52 (t, *J* = 5.1, 4H, **b**), 2.37 (s, 3H, **c**), 2.26 (t, *J* = 11.7, 2H, **C** ax), 2.22 – 2.15 (m, 2H, **B** eq), 1.72 – 1.56 (m, 2H, **B** ax). **¹³C NMR** (101 MHz, CDCl₃) δ 164.9, 159.8, 159.4, 156.9, 150.4, 144.3, 142.5, 129.1 (2C), 127.4 (2C), 123.8, 106.2, 103.2, 100.5, 62.7, 56.5, 55.9, 55.3 (2C), 52.7 (2C), 48.2, 46.4, 44.1 (2C), 32.3 (2C). **IR** (neat): 3281, 2941, 1659. **HRMS** (ESI): *m/z* found [M+H]⁺ 520.3021, C₂₈H₃₈N₇O₃ requires 520.3036.

4.1.4. Probe Synthesis

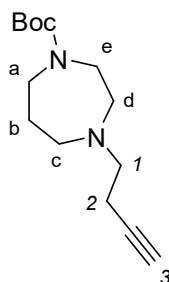
Compound 30: But-3-ynyl methanesulfonate



3-butyne-1-ol (1.5 mL, 20 mmol) was dissolved in DCM (25 mL) and NEt_3 (4.2 mL, 30 mmol) was added. A solution of methanesulfonyl chloride (2.3 mL, 30 mmol) in DCM (5.0 mL) was added dropwise and the mixture allowed to stir for 16 h at rt. HCl (2M, 20 mL) was added, and the aqueous layer extracted with DCM (20 mL). The organic layer was washed with HCl (1M, 30 mL), saturated sodium bicarbonate solution (30 mL) and brine (30 mL). The combined organic layers were dried over magnesium sulfate, concentrated *in vacuo* and taken on without further purification. The crude product was collected as a yellow-orange oil (2.96 g, 91%).

R_f 0.30 (EtOAc/pentane, 20:80). ^1H NMR (400 MHz, CDCl_3) δ 4.34 (t, $J = 6.7$, 2H, **1**), 3.08 (s, 3H, Ms CH_3), 2.69 (td, $J = 6.7, 2.7$, 2H, **2**), 2.09 (t, $J = 2.7$, 1H, **3**). ^{13}C NMR (101 MHz, CDCl_3) δ 78.61, 70.98, 67.07, 37.71, 19.78. IR (neat): 3287, 2940. HRMS (ESI): m/z found $[\text{M}+\text{H}]^+$ 149.0269, $\text{C}_5\text{H}_9\text{O}_3\text{S}$ requires 149.0272.

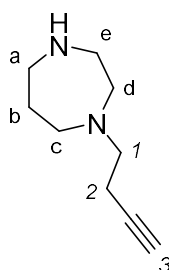
Compound 31: *tert*-butyl 4-(but-3-ynyl)-1,4-diazepane-1-carboxylate



Compound **30** (1.43g, 9.7 mmol) and 1-Boc-hexahydro-1,4-diazepine (2.0 mL, 10 mmol) were dissolved in acetonitrile (20 mL). Potassium carbonate (4.00 g, 29 mmol) and sodium iodide (750 mg, 5.0 mmol) were added, and the mixture heated to reflux for 3 h. The mixture was dissolved in DCM (80 mL), filtered, washed with water (100 mL) and brine (100 mL), dried over magnesium sulfate, concentrated *in vacuo* and purified by column chromatography eluting with DCM/MeOH (95:5). The product was collected as a yellow oil (1.47 g, 60%).

R_f 0.47 (DCM/MeOH, 95:5). $^1\text{H NMR}$ (400 MHz, CDCl_3) δ 3.54 – 3.41 (m, 4H, **a**, **e**), 2.80 – 2.64 (m, 6H, **c**, **d**, **1**), 2.43 – 2.30 (m, 2H, **2**), 2.04 – 1.97 (m, 1H, **3**), 1.91 – 1.78 (m, 2H, **b**), 1.48 (s, 9H, Boc (CH_3)₃).

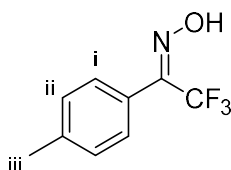
Compound 32: 1-(but-3-ynyl)-1,4-diazepane



Prepared by general procedure **E** with compound **31** (1.30 g, 5.2 mmol), the product was collected as an orange oil (730 mg, 93%).

R_f 0.21 (DCM/ NH_3 in MeOH (7N), 97:3). $^1\text{H NMR}$ (400 MHz, CDCl_3) δ 3.10 – 3.03 (m, 4H, **a**, **e**), 2.85 – 2.78 (m, 6H, **c**, **d**, **1**), 2.39 (td, $J = 7.4, 2.7$, 2H, **2**), 2.00 (t, $J = 2.7$, 1H, **3**), 1.88 (p, $J = 5.9$, 2H, **b**). $^{13}\text{C NMR}$ (101 MHz, CDCl_3) δ 82.7, 70.5, 69.2, 56.8, 53.7, 47.9, 46.1, 28.4, 17.3. **IR** (neat): 3288, 2950, 2830. **HRMS** (ESI): m/z found $[\text{M}+\text{H}]^+$ 153.1390, $\text{C}_9\text{H}_{17}\text{N}_2$ requires 153.1392.

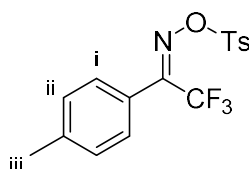
Compound 33: 2,2,2-trifluoro-1-p-tolyloxime



4'-Methyl-2,2,2-trifluoroacetophenone (2.50 g, 13 mmol) was dissolved in pyridine (25 mL). Hydroxylamine hydrochloride (2.77 g, 40 mmol) was added and the mixture heated to reflux for 4 h. The solvent was removed *in vacuo* and the residue dissolved in saturated aqueous citric acid solution (50 mL) and extracted with DCM (2 x 50 mL). The organic layers were washed with brine, dried over magnesium sulfate and concentrated *in vacuo*. The crude product was collected as a white solid (2.50 g, 93%) and taken on without purification.

R_f 0.51 (EtOAc/pentane, 20:80). $^1\text{H NMR}$ (400 MHz, CDCl_3) δ 7.45 (d, $J = 8.0$, 2H, i), 7.32 (d, $J = 8.0$, 2H, ii), 2.44 (s, 3H, iii).

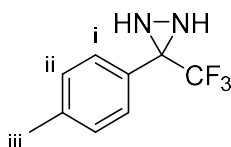
Compound 34: 2,2,2-trifluoro-1-p-tolyloxime O-tosyl oxime



Compound **33** (2.40 g, 12 mmol), NEt_3 (2.5 mL, 18 mmol) and 4-dimethylaminopyridine (720 mg, 5.9 mmol) were dissolved in anhydrous DCM (30 mL). The solution was cooled to 0 °C and p-toluenesulfonyl chloride (2.70 g, 14 mmol) was added. The mixture was allowed to warm slowly to rt and allowed to stir for 16 h. Brine (30 mL) was added and the organic layer extracted and washed with water (30 mL). The organic layer was then dried over magnesium sulfate, concentrated *in vacuo* and purified by column chromatography, eluting with pentane/EtOAc (10:90) to yield the product as a white solid (3.01 g, 71%).

R_f 0.85 (EtOAc/pentane, 20:80). $^1\text{H NMR}$ (400 MHz, CDCl_3) δ 7.94 – 7.89 (m, 2H, Ts Ar H), 7.43 – 7.38 (m, 2H, Ts Ar H), 7.34 (d, J = 8.3, 2H, i), 7.32 – 7.28 (m, 2H, ii), 2.50 (s, 3H, Ts CH₃), 2.43 (s, 3H, iii).

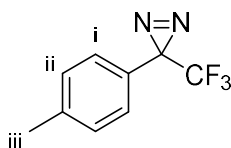
Compound 35: 3-p-tolyl-3-(trifluoromethyl)-diaziridine



Liquid NH_3 (6.0 mL) was condensed at -78°C . A solution of compound **34** (3.00 g, 8.4 mmol) in diethyl ether (25 mL) was added dropwise and the mixture allowed to stir for 2 h. The reaction was then slowly warmed to rt and left stirring for 16 h for the NH_3 to evaporate. The solution was then filtered and washed with diethyl ether (20 mL), and the filtrate was concentrated *in vacuo*. The product was then collected as a clear-white solid (1.41 g, 83%) and taken on without further purification.

R_f 0.53 (EtOAc/pentane, 20:80). $^1\text{H NMR}$ (400 MHz, CDCl_3) δ 7.52 (d, J = 8.1, 2H, i), 7.25 (d, J = 8.1, 2H, ii), 2.79 (d, J = 8.8, 1H, NH), 2.41 (s, 3H, iii), 2.21 (d, J = 9.6, 1H, NH). $^{13}\text{C NMR}$ (101 MHz, CDCl_3) δ 140.3, 129.9, 129.4 (2C), 128.0 (2C), 123.6 (q, J = 279), 57.9 (q, J = 37), 21.3.

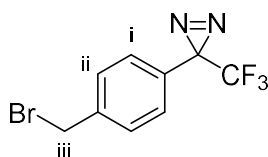
Compound 36: 3-p-tolyl-3-(trifluoromethyl)-3H-diazirine



NEt₃ (1.9 mL, 14 mmol) was added to a solution of compound **35** (1.35 g, 6.7 mmol) in DCM (20 mL) and the solution was cooled to 0 °C. Around one equivalent of iodine was added in small portions until the brown colour persisted. The solution was then allowed to stir for 1 h, washed with NaOH (1M, 20 mL), sodium thiosulfate solution (20 mL), water (20 mL) and brine (20 mL), before being dried over magnesium sulfate and concentrated *in vacuo* to yield the product as a yellow oil (929 mg, 69%).

R_f 0.50 (EtOAc/pentane, 20:80). ¹H NMR (400 MHz, CDCl₃) δ 7.22 (d, *J* = 8.3, 2H, i), 7.11 (d, *J* = 8.3, 2H, ii), 2.39 (s, 3H, iii). ¹⁹F NMR (471 MHz, CDCl₃) δ -66.6.

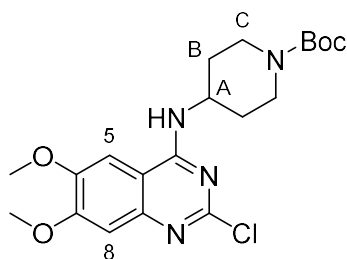
Compound 37: 3-(4-(bromomethyl)-phenyl)-3-(trifluoromethyl)-3*H*-diazirine



Compound **36** (475 mg, 2.4 mmol) was dissolved in carbon tetrachloride (10 mL), and *N*-bromosuccinimide (465 mg, 2.6 mmol) and azobisisobutyronitrile (50 mg, 0.30 mmol) were added. The solution was heated to reflux for 1 h before cooling to rt. Pentane (50 mL) was then added, and the solution filtered. The filtrate was concentrated *in vacuo* and the residue purified by column chromatography eluting with pentane/EtOAc (99:1) to yield the product as a yellow liquid (485 mg, 73%).

R_f 0.80 (EtOAc/pentane, 20:80). ¹H NMR (400 MHz, CDCl₃) δ 7.45 (d, *J* = 8.4, 2H, i), 7.19 (d, *J* = 8.4, 2H, ii), 4.49 (s, 2H, iii). ¹³C NMR (101 MHz, CDCl₃) δ 139.5, 129.5 (2C), 127.0, 126.9 (2C), 122.1 (q, *J* = 275), 40.0, 28.3 (q, *J* = 41). ¹⁹F NMR (471 MHz, CDCl₃) δ -65.2. HRMS (EI): *m/z* found [M-H]⁺ 276.9588, C₉H₅N₂BrF₃ requires 276.9588.

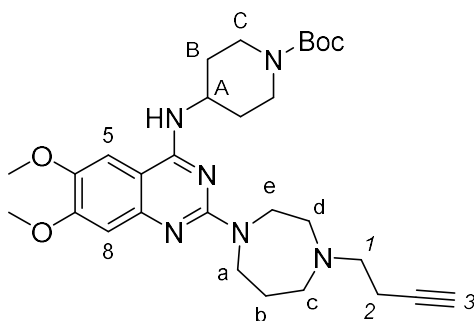
Compound 38: *tert*-butyl 4-(2-chloro-6,7-dimethoxyquinazolin-4-ylamino)-piperidine-1-carboxylate



Prepared by general procedure **A** with 6,7-dimethoxy-2,4-dichloroquinazoline (2.60 g, 10 mmol), 4-amino-1-Boc-piperidine (2.00 g, 10 mmol) and DIPEA (5.2 mL, 30 mmol). The crude product was purified by column chromatography eluting with a gradient of EtOAc/pentane (20:80 → 80:20) to yield the product as an off-white solid (723 mg, 17%).

R_f 0.38 (DCM/MeOH, 95:5). $^1\text{H NMR}$ (400 MHz, CDCl_3) δ 7.16 (s, 1H, **5**), 6.86 (s, 1H, **8**), 5.49 (d, $J = 7.6$, 1H, **NH**), 4.52 – 4.41 (m, 1H, **A**), 4.29 – 4.10 (m, 2H, **C** eq), 4.01 (s, 3H, **OCH₃**), 3.99 (s, 3H, **OCH₃**), 3.05 – 2.92 (m, 2H, **C** ax), 2.17 (d, $J = 12.1$, 2H, **B** eq), 1.49 (s, 9H, Boc (**CH₃**)₃), 1.57 – 1.43 (m, 2H, **B** ax). $^{13}\text{C NMR}$ (101 MHz, CDCl_3) δ 158.9, 156.1, 155.1, 154.7, 149.1, 148.2, 107.4, 106.6, 99.4, 79.8, 56.4, 56.3, 48.4 (2C), 42.3, 32.1 (2C), 28.5 (3C). **HRMS** (ESI): m/z found $[\text{M}+\text{H}]^+$ 423.1805, $\text{C}_{20}\text{H}_{28}\text{N}_4\text{O}_4\text{Cl}$ requires 423.1799.

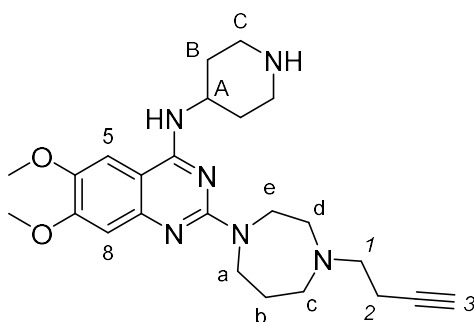
Compound 39: *tert*-butyl 4-(2-(4-(but-3-ynyl)-1,4-diazepan-1-yl)-6,7-dimethoxyquinazolin-4-ylamino)-piperidine-1-carboxylate



Prepared by general procedure **B** with compound **38** (300 mg, 0.71 mmol) and compound **32** (266 mg, 1.7 mmol). The crude product was purified by column chromatography eluting a gradient of DCM/NH₃ in MeOH (7N) (100:0 → 98:2) to yield the product as a pale-yellow oil (197 mg, 37%).

R_f 0.40 (DCM/NH₃ in MeOH (7N), 98:2). ¹H NMR (400 MHz, CDCl₃) δ 6.92 (s, 1H, **5**), 6.72 (s, 1H, **8**), 4.99 (s, 1H, **NH**), 4.31 – 4.21 (m, 1H, **A**), 4.20 – 4.10 (m, 2H, **e**), 3.98 (s, 3H, **OCH₃**), 3.95 (s, 3H, **OCH₃**), 3.89 (t, *J* = 6.3, 2H, **a**), 2.96 (t, *J* = 12.6, 2H, **C** eq), 2.89 – 2.84 (m, 2H, **c**), 2.84 – 2.75 (m, 2H, **1**), 2.75 – 2.67 (m, 2H, **d**), 2.39 (td, *J* = 7.3, 2.7, 2H, **2**), 2.20 – 2.14 (m, 4H, **C** ax, **b**), 1.98 (t, *J* = 2.7, 1H, **3**), 1.75 – 1.62 (m, 2H, **B** eq), 1.57 – 1.46 (m, 2H, **B** ax), 1.50 (s, 9H, Boc (**CH₃**)₃). ¹³C NMR (101 MHz, CDCl₃) δ 157.9, 154.8, 154.5, 145.2, 136.6, 129.0, 106.0, 102.7, 100.6, 83.0, 79.7, 69.0, 56.4, 56.3, 56.0, 55.68, 54.4, 48.5 (2C), 46.5, 45.8, 42.9, 32.1 (2C), 28.5 (3C), 27.9, 17.1. HRMS (ESI): *m/z* found [M+H]⁺ 539.3333, C₂₉H₄₃N₆O₄ requires 539.3346.

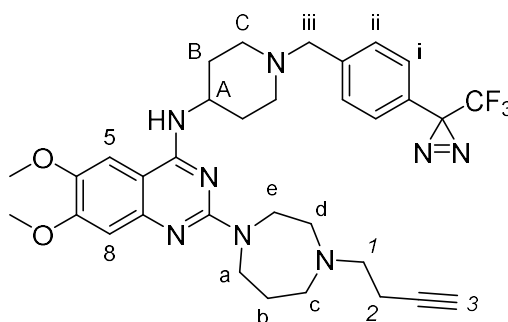
Compound 40: 2-(4-(but-3-ynyl)-1,4-diazepan-1-yl)-6,7-dimethoxy-*N*-(piperidin-4-yl)-quinazolin-4-amine



Prepared by general procedure **E** with compound **39** (175 mg, 0.32 mmol) to yield the product as an orange-yellow oil (64 mg, 46%).

R_f 0.42 (DCM/NH₃ in MeOH (7N), 95:5). **¹H NMR** (400 MHz, CDCl₃) δ 6.98 (s, 1H, **5**), 6.84 (s, 1H, **8**), 4.33 – 4.19 (m, 1H, **A**), 3.98 (s, 6H, OCH₃), 3.93 – 3.86 (m, 2H, **e**), 3.28 (d, J = 12.3, 2H, **C** eq), 2.91 – 2.83 (m, 4H, **a**, **c**), 2.83 – 2.77 (m, 2H, **1**), 2.71 (t, J = 5.2, 2H, **d**), 2.38 (td, J = 7.5, 2.7, 2H, **2**), 2.23 (d, J = 12.7, 2H, **C** ax), 1.98 (t, J = 2.6, 1H, **3**), 1.72 – 1.60 (m, 4H, **B** eq, **b**), 1.35 – 1.25 (m, 2H, **B** ax). **¹³C NMR** (101 MHz, CDCl₃) δ 157.9, 154.5, 145.3, 140.2, 136.3, 110.0, 105.6, 102.7, 83.0, 69.0, 56.4, 56.2, 56.1, 55.6, 54.3, 48.2 (2C), 46.6, 45.9, 45.2, 27.8 (2C), 27.6, 17.1. **IR** (neat): 3293, 2935, 2115. **HRMS** (ESI): m/z found [M+H]⁺ 439.2830, C₂₄H₃₅N₆O₂ requires 439.2821.

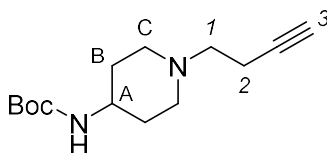
Probe 1: 2-(4-(but-3-ynyl)-1,4-diazepan-1-yl)-6,7-dimethoxy-*N*-(1-(4-(3-(trifluoromethyl)-3*H*-diazirin-3-yl)-benzyl)-piperidin-4-yl)-quinazolin-4-amine



Compound **40** (35 mg, 0.080 mmol) was dissolved in MeOH (5.0 mL). A solution compound **37** (26 mg, 0.090 mmol) in THF (5.0 mL) was added, followed by potassium carbonate (25 mg, 0.18 mmol). The mixture was allowed to stir at rt for 18 h before being concentrated *in vacuo*. The residue was dissolved in EtOAc (50 mL) and washed with saturated sodium bicarbonate solution (50 mL). The organic layer was then dried over magnesium sulfate, concentrated *in vacuo* and purified by column chromatography, eluting with DCM/NH₃ in MeOH (97.5:2.5) to yield the product as an off-white solid (12 mg, 24%) which was $\geq 95\%$ pure by LC-MS analysis.

R_f 0.40 (DCM/NH₃ in MeOH (7N), 97.5:2.5). **m.p.** 52-54 °C. **¹H NMR** (400 MHz, CDCl₃) δ 7.41 (d, *J* = 8.3, 2H, **i**), 7.17 (d, *J* = 8.3Hz, 2H, **ii**), 6.91 (s, 1H, **5**), 6.71 (s, 1H, **8**), 4.95 (s, 1H, **NH**), 4.18 – 4.07 (m, 1H, **A**), 4.03 – 3.92 (m, 2H, **e**), 3.98 (s, 3H, OCH₃), 3.96 (s, 3H, OCH₃), 3.92 – 3.84 (m, 2H, **a**), 3.57 (s, 2H, **iii**), 2.92 – 2.83 (m, 4H, **C** eq, **c**), 2.79 (t, *J* = 7.6, 2H, **1**), 2.70 (t, *J* = 5.5, 2H, **d**), 2.38 (td, *J* = 7.6, 2.7, 2H, **2**), 2.26 – 2.12 (m, 4H, **C** ax, **b**), 2.09 – 1.86 (m, 2H, **B** eq), 1.97 (t, *J* = 2.7, 1H, **3**), 1.73 – 1.58 (m, 2H, **B** ax). **¹³C NMR** (125 MHz, CDCl₃) δ 160.8, 158.0, 154.5, 150.3, 146.9, 140.5, 129.3 (2C), 127.9, 126.4 (2C), 122.1 (q, *J* = 284), 105.8, 102.5, 100.7, 82.8, 69.1, 62.4, 56.5, 56.3, 55.6, 55.4, 54.3, 52.6 (2C), 48.7, 46.6, 32.1 (2C), 29.1, 28.4 (q, *J* = 41), 27.7, 17.1. **¹⁹F NMR** (471 MHz, CDCl₃) δ -65.2. **IR** (neat): 3301, 2941. **HRMS** (ESI): *m/z* found [M+H]⁺ 637.3215, C₃₃H₄₀N₈O₂F₃ requires 637.3226.

Compound 41: *tert*-butyl 1-(but-3-ynyl)-piperidin-4-ylcarbamate

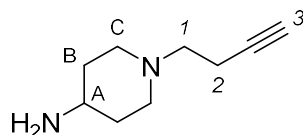


4-(*N*-Boc-amino)-piperidine (800 mg, 4.0 mmol), NEt₃ (0.60 mL, 4.3 mmol), potassium iodide (33 mg, 0.20 mmol) and compound **30** (590 mg, 4.0 mmol) were dissolved in DMSO (12 mL). The mixture was then allowed to stir at rt for 16 h, dissolved in water (50 mL) and extracted with diethyl ether (50 mL). The organic layer was washed with brine (50 mL), dried over magnesium sulfate, concentrated *in vacuo* and purified by column chromatography, eluting with DCM/MeOH (97.5:2.5) to yield the product as an off-white solid (409 mg, 41%).

R_f 0.38 (DCM/MeOH, 97.5:2.5). **¹H NMR** (400 MHz, CDCl₃) δ 4.51 – 4.38 (m, 1H, **A**), 3.49 (s, 1H, **NH**), 2.86 (d, *J* = 11.6, 2H, **C** eq), 2.61 (t, *J* = 7.6, 2H, **1**), 2.39 (td, *J* = 7.6, 2.7, 2H, **2**), 2.16 (td, *J* = 11.6, 2.6, 2H, **C** ax), 2.00 (t, *J* = 2.7, 1H, **3**), 1.95 (d, *J* = 12.7, 2H, **B** eq),

1.47 (s, 9H, Boc (CH₃)₃), 1.45 – 1.34 (m, 2H, **B** ax). **HRMS** (ESI): *m/z* found [M+H]⁺ 253.1925, C₁₄H₂₅N₂O₂ requires 253.1916.

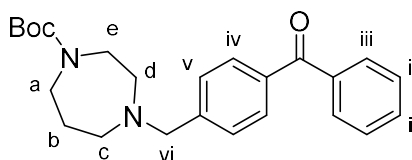
Compound 42: 1-(but-3-ynyl)-piperidin-4-amine



Prepared by general procedure **E** with *tert*-butyl compound **41** (400 mg, 2.0 mmol), the product was collected as a yellow oil (192 mg, 64%).

¹H NMR (400 MHz, CDCl₃) δ 2.91 (dt, *J* = 12.0, 3.7, 2H, **C** eq), 2.79 – 2.69 (m, 1H, **A**), 2.62 (t, *J* = 7.6, 2H, **1**), 2.40 (td, *J* = 7.6, 2.7, 2H, **2**), 2.12 (td, *J* = 12.0, 2.9, 2H, **C** ax), 2.00 (t, *J* = 2.7, 1H, **3**), 1.92 – 1.81 (m, 2H, **B** eq), 1.55 – 1.38 (m, 2H, **B** ax). **¹³C NMR** (101 MHz, CDCl₃) δ 82.8, 69.1, 56.9, 52.0 (2C), 48.6, 35.1, 16.9 (2C). **HRMS** (ESI): *m/z* found [M+H]⁺ 153.1384, C₉H₁₇N₂ requires 153.1392.

Compound 43: *tert*-butyl 4-((4-benzoylphenyl)-methyl)-1,4-diazepane-1-carboxylate

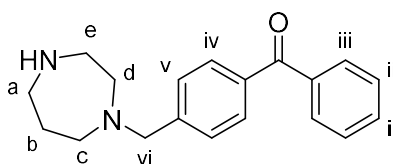


A solution of 1-Boc-hexahydro-1,4-diazepine (0.70 mL, 3.6 mmol), 4-(Bromomethyl)-benzophenone (1.00 g, 3.6 mmol) and NEt₃ (0.60 mL, 4.3 mmol) in DCM (25 mL) was allowed to stir at room temperature for 24 h. Water (50 mL) was added and the aqueous layer extracted with DCM (50 mL). The combined organic layers were dried over magnesium

sulfate, concentrated *in vacuo* and purified by column chromatography eluting with DCM/MeOH (96:4) to yield the product as a white solid (1.10 g, 77%).

R_f 0.34 (DCM/MeOH, 96:4). $^1\text{H NMR}$ (400 MHz, CDCl_3) δ 7.86 – 7.76 (m, 4H, **iii**, **iv**), 7.65 – 7.58 (m, 1H, **i**), 7.54 – 7.45 (m, 4H, **ii**, **v**), 3.73 (s, 2H, **vi**), 3.58 – 3.51 (m, 2H, **e**), 3.51 – 3.44 (m, 2H, **a**), 2.76 – 2.62 (m, 4H, **c**, **d**), 1.93 – 1.80 (m, 2H, **b**), 1.49 (s, 9H, Boc (CH_3)₃). $^{13}\text{C NMR}$ (101 MHz, CDCl_3) δ 196.5, 155.6, 144.5, 137.8, 136.4, 132.3, 130.2 (2C), 130.0 (2C), 128.5 (2C), 128.3 (2C), 79.3, 62.0, 56.3, 53.4, 46.8, 45.3, 28.5 (3C), 28.1. **HRMS** (ESI): m/z found $[\text{M}+\text{H}]^+$ 395.2346, $\text{C}_{24}\text{H}_{31}\text{N}_2\text{O}_3$ requires 395.2335.

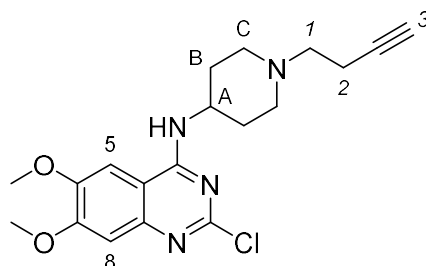
Compound 44: (4-((1,4-diazepan-1-yl)-methyl)-phenyl)-(phenyl)-methanone



Prepared by general procedure **E** with compound **43** (1.05 g, 2.7 mmol), to yield the product as a brown oil (439 mg, 56%).

$^1\text{H NMR}$ (400 MHz, CDCl_3) δ 7.85 – 7.76 (m, 4H, **iii**, **iv**), 7.65 – 7.58 (m, 1H, **i**), 7.54 – 7.45 (m, 4H, **ii**, **v**), 3.77 (s, 2H, **vi**), 3.11 – 3.05 (m, 2H, **e**), 3.04 – 2.96 (m, 2H, **a**), 2.79 – 2.67 (m, 4H, **c**, **d**), 1.91 – 1.77 (m, 2H, **b**). $^{13}\text{C NMR}$ (101 MHz, CDCl_3) δ 196.5, 144.6, 137.8, 136.4, 132.3, 130.2 (2C), 130.0 (2C), 128.5 (2C), 128.3 (2C), 62.5, 54.7, 53.4, 48.6, 46.8, 31.3. **HRMS** (ESI): m/z found $[\text{M}+\text{H}]^+$ 295.1811, $\text{C}_{19}\text{H}_{23}\text{N}_2\text{O}$ requires 295.1810.

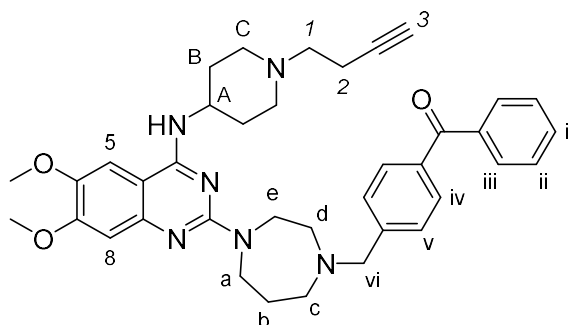
Compound 45: *N*-(1-(but-3-ynyl)-piperidin-4-yl)-2-chloro-6,7-dimethoxyquinazolin-4-amine



Prepared by general procedure **A** with 6,7-dimethoxy-2,4-dichloroquinazoline (259 mg, 1.0 mmol), compound **42** (152 mg, 1.0 mmol) and DIPEA (0.52 mL 3.0 mmol). The crude product was purified by column chromatography eluting with DCM/NH₃ in MeOH (7N) (97:3) to yield the product as an off-white solid (128 mg, 34%).

R_f 0.72 (DCM/NH₃ in MeOH (7N), 97:3). **¹H NMR** (400 MHz, CDCl₃) δ 7.17 (s, 1H, **5**), 6.78 (s, 1H, **8**), 5.27 (d, *J* = 7.7, 1H, **NH**), 4.35 – 4.24 (m, 1H, **A**), 4.04 (s, 2H, **OCH₃**), 4.01 (s, 3H, **OCH₃**), 2.98 (d, *J* = 11.5, 2H, **C** eq), 2.68 (t, *J* = 7.4, 2H, **1**), 2.44 (td, *J* = 7.4, 2.6, 2H, **2**), 2.34 (t, *J* = 11.5, 2H, **C** ax), 2.20 (d, *J* = 12.4, 2H, **B** eq), 2.03 (t, *J* = 2.6, 1H, **3**), 1.69 – 1.55 (m, 2H, **B** ax). **¹³C NMR** (101 MHz, CDCl₃) δ 159.0, 156.2, 155.0, 149.1, 148.1, 107.4, 106.6, 99.4, 82.8, 69.1, 56.9, 56.4, 56.3, 52.1 (2C), 48.2, 32.2 (2C), 17.0. **IR** (neat): 3388, 3246, 2934, 2116. **HRMS** (ESI): *m/z* found [M+H]⁺ 375.1572, C₁₉H₂₄N₄O₂Cl requires 375.1588.

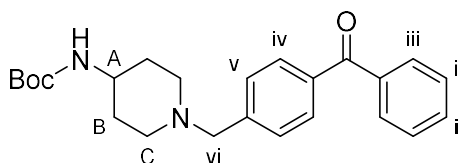
Probe 2: (4-((4-(4-((1-(but-3-yn-1-yl)-piperidin-4-yl)-amino)-6,7-dimethoxyquinazolin-2-yl)-1,4-diazepan-1-yl)-methyl)-phenyl)-(phenyl)methanone



Prepared by general procedure **B** with compound **45** (85 mg, 0.23 mmol) and compound **44** (200 mg, 0.68 mmol). The crude product was purified by column chromatography eluting with a gradient of DCM/MeOH NH₃ solution (7N) (100:0 → 97.5:2.5) to yield the product as a dark brown oil (78 mg, 54%) which was ≥95% pure by LC-MS analysis.

R_f 0.33 (DCM/NH₃ in MeOH (7N), 98:2). ¹H NMR (400 MHz, CDCl₃) δ 7.86 – 7.75 (m, 4H, **iii**, **iv**), 7.65 – 7.57 (m, 1H, **i**), 7.54 – 7.44 (m, 4H, **ii**, **v**), 6.91 (s, 1H, **5**), 6.73 (s, 1H, **8**), 4.98 (d, *J* = 7.1, 1H, **NH**), 4.15 – 4.05 (m, 1H, **A**), 4.02 – 3.99 (m, 2H, **e**) 3.97 (s, 3H, OCH₃), 3.95 (s, 3H, OCH₃), 3.94 – 3.87 (m, 2H, **a**), 3.74 (s, 2H, **vi**), 3.01 – 2.92 (m, 2H, **C** eq), 2.87 – 2.79 (m, 2H, **c**), 2.72 – 2.61 (m, 4H, **1**, **d**), 2.42 (td, *J* = 7.5, 2.7, 2H, **2**), 2.29 – 2.12 (m, 4H, **C** eq, **B** eq), 2.01 (t, *J* = 2.7, 1H, **3**), 1.69 – 1.55 (m, 2H, **b**), 1.36 – 1.22 (m, 2H, **B** ax). ¹³C NMR (101 MHz, CDCl₃) δ 196.5, 158.1, 154.4, 149.8, 149.7, 145.1, 144.8, 137.8, 136.3, 132.3, 130.2 (2C), 130.0 (2C), 128.5 (2C), 128.2 (2C), 106.1, 102.7, 100.7, 82.8, 69.1, 61.9, 57.0, 56.4, 56.0, 55.0, 53.4, 52.3 (2C), 48.2, 46.6, 46.0, 32.2 (2C), 28.1, 17.1. IR (neat): 2939, 1648 (C=O). HRMS (ESI): *m/z* found (M+H)⁺ 633.3562, C₃₈H₄₅N₆O₃ requires 633.3553.

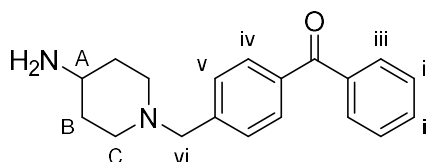
Compound 46: *tert*-butyl (1-((4-benzoylphenyl)-methyl)-piperidin-4-yl)-carbamate



A solution of 4-(*N*-Boc-amino)-piperidine (728 mg, 3.6 mmol), 4-(bromomethyl)-benzophenone (1.00 g, 3.6 mmol) and NEt₃ (0.60 mL, 4.3 mmol) in DCM (25 mL) was allowed to stir at room temperature for 24 h. Water (50 mL) was added and the aqueous layer extracted with DCM (50 mL). The combined organic layers dried over magnesium sulfate, concentrated *in vacuo* and purified by column chromatography eluting with DCM/MeOH (97:3) to yield the product as a white solid (1.09 g, 76%).

R_f 0.35 (DCM/MeOH, 97:3). **¹H NMR** (400 MHz, CDCl₃) δ 7.84 – 7.77 (m, 4H, **iii**, **iv**), 7.66 – 7.57 (m, 1H, **i**), 7.53 – 7.43 (m, 4H, **ii**, **v**), 4.46 (s, 1H, **NH**), 3.58 (s, 2H, **vi**), 3.55 – 3.46 (m, 1H, **A**), 2.84 (d, *J* = 11.7, 2H, **C** eq), 2.16 (t, *J* = 11.3, 2H, **C** ax), 1.95 (d, *J* = 12.6, 2H, **B** eq), 1.64 – 1.55 (m, 2H, **B** ax), 1.47 (s, 9H, **CH₃**). **HRMS** (ESI): *m/z* found (M+H)⁺ 395.2330, C₂₄H₃₁N₂O₃ requires 395.2335.

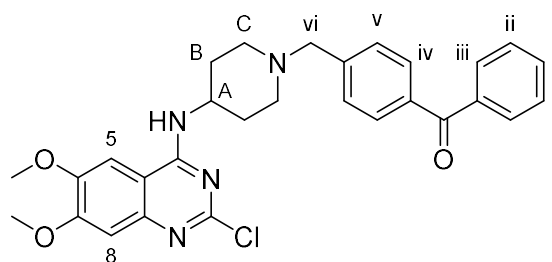
Compound 47: (4-((4-aminopiperidin-1-yl)-methyl)-phenyl)-(phenyl)methanone



Prepared by general procedure **E** with compound **46** (1.00 g, 2.5 mmol), to yield the product as a yellowish-brown oil (583 mg, 78%).

¹H NMR (400 MHz, CDCl₃) δ 7.85 – 7.75 (m, 4H, **iii**, **iv**), 7.64 – 7.57 (m, 1H, **i**), 7.53 – 7.42 (m, 4H, **ii**, **v**), 3.58 (s, 2H, **vi**), 2.93 – 2.82 (m, 2H, **C** eq), 2.77 – 2.67 (m, 1H, **A**), 2.15 – 2.01 (m, 2H, **C** ax), 1.84 (ddd, *J* = 13.4, 5.1, 2.8, 2H, **B** eq), 1.56 – 1.36 (m, 2H, **B** ax). **¹³C NMR** (101 MHz, CDCl₃) δ 196.5, 143.8, 137.8, 136.3, 132.3, 130.2 (2C), 130.0 (2C), 128.8 (2C), 128.3 (2C), 62.7, 52.5 (2C), 48.7, 35.5 (2C). **HRMS** (ESI): *m/z* found (M+H)⁺ 295.1821, C₁₉H₂₃N₂O requires 295.1810.

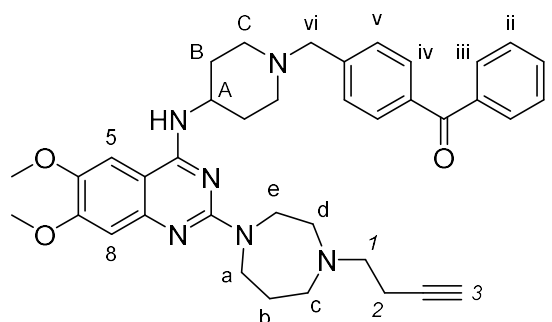
Compound 48: (4-((4-((2-chloro-6,7-dimethoxyquinazolin-4-yl)-amino)-piperidin-1-yl)-methyl)-phenyl)-(phenyl)methanone



Prepared by general procedure **A** with 6,7-dimethoxy-2,4-dichloroquinazoline (484 mg, 1.9 mmol), compound **47**, (550 mg, 1.9 mmol) and NEt₃ (0.80 mL, 5.7 mmol). The crude product was purified by column chromatography eluting with DCM/NH₃ in MeOH (7N) (97:3) to yield the product as a pale-yellow solid (500 mg, 52%).

R_f 0.48 (DCM/NH₃ in MeOH (7N), 97:3). **¹H NMR** (400 MHz, CDCl₃) δ 7.87 – 7.78 (m, 4H, **iii**, **iv**), 7.66 – 7.59 (m, 1H, **i**), 7.55 – 7.46 (m, 4H, **ii**, **v**), 7.17 (s, 1H, **5**), 6.79 (s, 1H, **8**), 5.32 (d, *J* = 7.8, 1H, **NH**), 4.39 – 4.28 (m, 1H, **A**), 4.03 (s, 3H, **OCH₃**), 4.00 (s, 3H, **OCH₃**), 3.66 (s, 2H, **vi**), 2.95 (d, *J* = 11.7, 2H, **C** eq), 2.32 (t, *J* = 11.3, 2H, **C** ax), 2.19 (d, *J* = 11.5, 2H, **B** eq), 1.75 – 1.63 (m, 2H, **B** ax). **¹³C NMR** (101 MHz, CDCl₃) δ 196.6, 159.1, 156.2, 155.0, 149.1, 148.1, 143.6, 137.7, 136.5, 132.4, 130.2 (2C), 130.0 (2C), 128.8 (2C), 128.3 (2C), 107.4, 106.7, 99.5, 62.6, 56.4, 56.3, 52.5 (2C), 48.3, 32.3 (2C). **HRMS** (ESI): *m/z* found (M+H)⁺ 517.1996, C₂₉H₃₀N₄O₃Cl requires 517.2006.

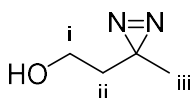
Probe 3: (4-((4-((2-(4-(but-3-yn-1-yl)-1,4-diazepan-1-yl)-6,7-dimethoxyquinazolin-4-yl)-amino)-piperidin-1-yl)-methyl)-phenyl)-(phenyl)methanone



Prepared by general procedure **B** with compound **48** (215 mg, 0.42 mmol) and compound **32** (190 mg, 1.3 mmol). The crude product was purified by column chromatography eluting with DCM/NH₃ in MeOH (7N) (99:1) to yield the product as a pale brown solid (63 mg, 24%) which was ≥95% pure by LC-MS analysis.

R_f 0.21 (DCM/NH₃ in MeOH (7N), 98:2). **¹H NMR** (400 MHz, CDCl₃) δ 7.87 – 7.79 (m, 4H, **iii**, **iv**), 7.66 – 7.59 (m, 1H, **i**), 7.55 – 7.47 (m, 4H, **ii**, **v**), 6.90 (s, 1H, **5**), 6.71 (s, 1H, **8**), 4.97 (s, 1H, **NH**), 4.21 – 4.09 (m, 1H, **A**), 3.99 – 3.95 (m, 2H, **e**), 3.98 (s, 3H, **OCH₃**), 3.96 (s, 3H, **OCH₃**), 3.89 (t, *J* = 6.3, 2H, **a**), 3.65 (s, 2H, **vi**), 2.95 (d, *J* = 11.5, 2H, **C** eq), 2.89 – 2.83 (m, 2H, **c**), 2.84 – 2.75 (m, 2H, **1**), 2.74 – 2.68 (m, 2H, **d**), 2.38 (td, *J* = 7.5, 2.6, 2H, **2**), 2.31 – 2.16 (m, 4H, **C** ax, **B** eq), 1.98 (t, *J* = 2.6, 1H, **3**), 1.73 – 1.60 (m, 4H, **B** ax, **b**). **¹³C NMR** (101 MHz, CDCl₃) δ 196.5, 158.1, 154.4, 151.1, 150.4, 148.3, 143.8, 137.8, 136.4, 132.3, 130.2 (2C), 130.0 (2C), 128.7 (2C), 128.3 (2C), 106.2, 106.1, 100.7, 91.6, 69.0, 62.8, 56.4, 56.3, 56.0, 55.7, 54.3, 52.8 (2C), 48.3, 46.4, 45.7, 32.4 (2C), 27.9, 17.1. **IR** (neat): 2935, 1649 (C=O). **HRMS** (ESI): *m/z* found (M+H)⁺ 633.3562, C₃₈H₄₅N₆O₃ requires 633.3553.

Compound 49: 2-(3-methyl-3*H*-diaziren-3-yl)-ethanol

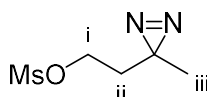


NH₃ (20 mL) was condensed at -78 °C into a flask containing 4-hydroxy-2-butanone (2.0 mL, 23 mmol) and the mixture was allowed to stir at -78 °C for 4 h. Hydroxylamine-O-sulfonic acid (3.15 g, 28 mmol) was dissolved in MeOH (20 mL) and the solution added to the reaction over 30 min. The mixture was allowed to stir at -78 °C, slowly warmed to rt and allowed to stir for 16 h. The slurry was diluted with MeOH (30 mL), filtered and washed with MeOH (2 x 15 mL). NEt₃ (6.5 mL, 47 mmol) was added to the filtrate, followed by iodine until the reddish-brown colour persisted, and the solution was allowed to stir at rt for 2 h. The solvent was removed *in vacuo*, and the residue diluted with diethyl ether (150 mL), washed with aqueous sodium thiosulfate solution (10%, 150 mL) and HCl (1M, 100 mL), dried over magnesium sulfate and concentrated *in vacuo* to yield the product as a yellow solid (1.08g, 46%). The product was taken on without further purification.

¹H NMR (400 MHz, CDCl₃) δ 3.56 (t, *J* = 6.3, 2H, i), 1.67 (t, *J* = 6.2, 2H, ii), 1.10 (s, 3H, iii).

¹³C NMR (101 MHz, CDCl₃) δ 57.8, 37.0, 24.2, 20.3.

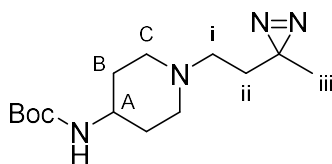
Compound 50: 2-(3-methyl-3*H*-diaziren-3-yl)-ethyl methanesulfonate



A solution of compound **49** (1.0g, 10 mmol) in DCM (40 mL) was cooled to 0 °C. Methanesulfonyl chloride (1.2 mL, 16 mmol) and NEt₃ (2.1 mL, 15 mmol) were added and the solution allowed to warm to rt and allowed to stir for 5 h. The solution was washed with HCl (1M, 50 mL), saturated aqueous sodium hydrogen carbonate solution (50 mL) and brine (50 mL). The organic layer was then dried over magnesium sulfate and concentrated *in vacuo* to yield the product as a yellow solid (1.08g, 46%). The product was taken on without further purification.

¹H NMR (400 MHz, CDCl₃) δ 4.16 (t, *J* = 6.2, 2H, **i**), 3.09 (s, 3H, Ms **CH₃**), 1.82 (t, *J* = 6.3, 2H, **ii**), 1.13 (s, 3H, **iii**). **¹³C NMR** (101 MHz, CDCl₃) δ 64.4, 52.6, 37.7, 34.3, 19.9. **HRMS** (EI): *m/z* found [M+NH₄]⁺ 196.0765, C₅H₁₄N₃O₃ requires 196.0756.

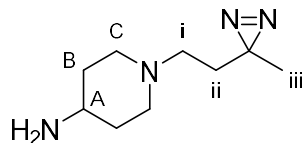
Compound 51: *tert*-butyl (1-(2-(3-methyl-3*H*-diaziren-3-yl)-ethyl)-piperidin-4-yl)-carbamate



Compound **50** (900 mg, 5.1 mmol), 4-(*N*-Boc-amino)-piperidine (1.01 g, 5.0 mmol), potassium carbonate (2.11 g, 15 mmol) and sodium iodide (382 mg, 2.5 mmol) were dissolved in acetonitrile (50 mL), and the mixture heated to reflux for 3 h. The mixture was dissolved in DCM (80 mL), filtered, washed with water (100 mL) and brine (100 mL), dried over magnesium sulfate, concentrated *in vacuo* and purified by column chromatography eluting with DCM/MeOH (97:3). The product was collected as a pale orange solid (921 mg, 65%).

R_f 0.44 (DCM/MeOH, 97:3). **¹H NMR** (400 MHz, CDCl₃) δ 3.55 – 3.39 (m, 1H, **A**), 2.85 – 2.73 (m, 2H, **C** eq), 2.30 – 2.21 (m, 2H, **i**), 2.09 – 2.01 (m, 2H, **C** ax), 1.94 (d, *J* = 12.6, 2H, **B** eq), 1.54 – 1.49 (m, 2H, **ii**), 1.46 (s, 9H, Boc (**CH₃**)₃), 1.42 – 1.36 (m, 2H, **B** ax), 1.04 (s, 3H, **iii**). **¹³C NMR** (101 MHz, CDCl₃) δ 155.2, 79.3 53.4, 52.8 (2C), 52.3, 47.7, 32.6 (2C), 32.3, 28.4 (3C), 20.0.

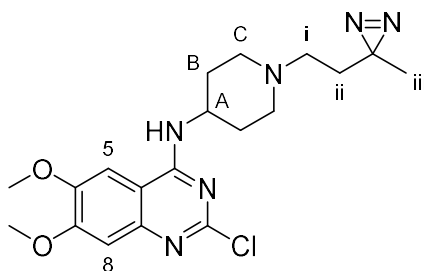
Compound 52: 1-(2-(3-methyl-3*H*-diaziren-3-yl)-ethyl)-piperidin-4-amine



Prepared by general procedure **E** with compound **51** (900 mg, 3.2 mmol) to yield the product as a brown oil (552 mg, 95%).

¹H NMR (400 MHz, CDCl₃) δ 2.87 – 2.77 (m, 2H, **C** eq), 2.72 – 2.60 (m, 1H, **A**), 2.30 – 2.22 (m, 2H, **i**), 2.06 – 1.93 (m, 2H, **C** ax), 1.87 – 1.77 (m, 2H, **B** eq), 1.55 – 1.48 (m, 2H, **ii**), 1.46 – 1.33 (m, 2H, **B** ax), 1.04 (s, 3H, **iii**). **¹³C NMR** (101 MHz, CDCl₃) δ 53.4, 52.8, 52.4 (2C), 48.7, 35.9 (2C), 32.3, 20.0.

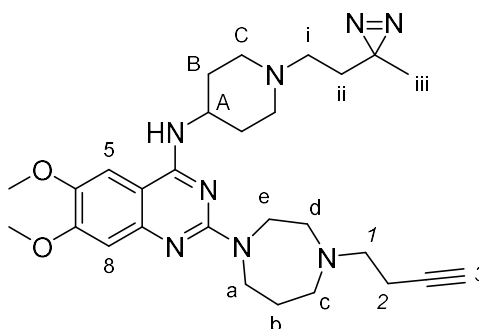
Compound 53: 2-chloro-6,7-dimethoxy-*N*-(1-(2-(3-methyl-3*H*-diaziren-3-yl)-ethyl)-piperidin-4-yl)-quinazolin-4-amine



Prepared by general procedure **A** with 6,7-dimethoxy-2,4-dichloroquinazoline (711 mg, 2.7 mmol) compound **52** (500 mg, 2.7 mmol) and DIPEA (1.5 mL, 8.3 mmol). The crude product was purified by column eluting with a gradient of DCM/NH₃ in MeOH (7N) (99:1 → 97:3) to yield the product as a pale-yellow solid (168 mg, 15%).

R_f 0.70 (DCM/NH₃ in MeOH (7N), 97:3). **¹H NMR** (400 MHz, CDCl₃) δ 7.13 (s, 1H, **5**), 6.84 (s, 1H, **8**), 5.47 (d, J = 7.8, 1H, **NH**), 4.35 – 4.23 (m, 1H, **A**), 4.00 (s, 3H, **OCH₃**), 3.97 (s, 3H, **OCH₃**), 2.93 – 2.82 (m, 2H, **C** eq), 2.36 – 2.28 (m, 2H, **i**), 2.23 – 2.13 (m, 4H, **C** ax, **B** eq), 1.69 – 1.57 (m, 2H, **ii**), 1.56 – 1.50 (m, 2H, **B** ax), 1.06 (s, 3H **iii**). **¹³C NMR** (101 MHz, CDCl₃) δ 159.1, 156.2, 155.0, 149.0, 148.1, 107.3, 106.7, 99.6, 77.2, 56.4, 56.3, 52.8, 52.2 (2C), 48.3, 32.2 (2C), 25.0, 20.0.

Probe 4: 2-(4-(but-3-yn-1-yl)-1,4-diazepan-1-yl)-6,7-dimethoxy-*N*-(1-(2-(3-methyl-3*H*-diaziren-3-yl)-ethyl)-piperidin-4-yl)-quinazolin-4-amine



Compound **53** (100 mg, 0.25 mmol) and compound **32** (120 mg, 0.79 mmol) were dissolved in toluene (5.0 mL) and heated to reflux for 18 h. The reaction mixture was then purified by column chromatography eluting with a gradient of DCM/NH₃ in MeOH (7N) (100:0 → 98:2) to yield the product as a pale-yellow oil (5.0 mg, 4%) which was $\geq 95\%$ pure by LC-MS analysis.

¹H NMR (400 MHz, MeOD) δ 7.44 (s, 1H, **5**), 6.94 (s, 1H, **8**), 4.17-4.13 (m, 2H, **e**), 3.97 (m, 1H, **A**), 3.93 (s, 3H, **OCH₃**), 3.91 (s, 3H, **OCH₃**), 3.86 (t, J = 6.3, 2H, **a**), 3.06-2.98 (m, 2H, **C** eq), 2.89 (t, J = 5.0, 2H, **1**), 2.80-2.70 (m, 6H, **C** ax, **c**, **d**), 2.47-2.34 (m, 4H, **B** eq, **2**), 2.28 (t, J = 2.7, 1H, **3**), 2.19-2.08 (m, 2H, **i**), 2.08-1.95 (m, 2H, **b**), 1.77-1.73 (m, 2H, **ii**), 1.62-1.54 (m, 2H, **B** ax), 1.06 (s, 3H, **iii**). **HRMS** (ESI) m/z found $[M+H]^+$ 521.3370, C₂₈H₄₁N₈O₂ requires 521.3352

4.2. Biological Testing

4.2.1. General Procedures

A Milli-Q Millipore purification system was used to give ultrapure 18.2 mΩ laboratory water. PBS was purchased from Invitrogen. Other reagents were purchased from commercial sources.

4.2.1.1. Buffer Composition

Table 10: Composition of buffers used.

Buffer	Composition
Coomassie Destaining Solution	Acetic Acid 10%, MeOH 50%
Coomassie Staining Solution	Coomassie Brilliant Blue R-250 0.1%, Acetic Acid 10%, MeOH 50%
Culture Media	Haematocrit 2%, Initial Parasitemia 0.5–0.8%, RPMI 1640, Albumax 0.5%, Sodium Bicarbonate 0.25%, Hypoxanthine 50 mg L ⁻¹ , Gentamicin 25 mg L ⁻¹
Cytoplasm Lysis Buffer	HEPES 10 mM, KCl, 10 mM, NP40 0.65%, DTT 1mM and EDTA-free protease inhibitors (Roche cOmplete)
Dynabead Conjugation Buffer	20 mM Na ₃ PO ₄ , 0.15 M NaCl
Dynabead Washing Buffer	0.05% Tween20, 5 mg mL ⁻¹ BSA in PBS
Gel Fixing Solution	Acetic Acid 10%
Nitrogen Lysis Buffer	Roche cOmplete EDTA-free protease inhibitors, 1 tablet in 40 mL PBS
Nuclear Extraction Buffer	HEPES 20 mM, KCl 600 mM, MgCl ₂ 2 mM, ZnCl ₂ 25 μM, DTT 1 mM and EDTA-free protease inhibitors (Roche cOmplete)
PBST	0.05% Tween20 in PBS
Saponin Lysis Buffer I	0.075% saponin in PBS
Saponin Lysis Buffer II	0.15% saponin in RPMI-16
SYBR Green I Lysis Buffer	Tris pH 7.5 20mM, EDTA 5mM, saponin 0.008%, Triton X-100 0.08%, 2x SYBR Green I
Western 2° Antibody Buffer	0.05% Tween20, 10% Skim Milk Powder in PBS
Western Blocking Buffer	0.05% Tween20, 5% Bovine Serum Albumin (BSA) in PBS

4.2.2. *P. falciparum* Growth and Proliferation Assays

Performed by the Scherf Lab at the Pasteur Institute, Paris.^{103-104,164}

Compounds were tested against drug sensitive *P. falciparum* 3D7 strain parasites using a three-day SYBR Green I based assay. Parasites were cultured at 2% hematocrit with an initial parasitemia of 0.5–0.8% in RPMI 1640 containing 0.5% Albumax. Compounds were initially screened at 2 μ M in duplicate wells in 100 μ L cultures in a 96-well format. Parasites were allowed to grow with compounds for three days. To each well 100 μ L of SYBR Green I lysis buffer (20mM Tris pH 7.5, 5 mM EDTA, 0.008% saponin, 0.08% Triton X-100, 2x SYBR Green I) was added. Lysed parasite samples were measured for total SYBR Green I fluorescence (ex: 488nm, em: 520nm). Compounds were then tested at a range of concentrations. Data was fitted to a standard inhibition curve with a variable Hill slope.

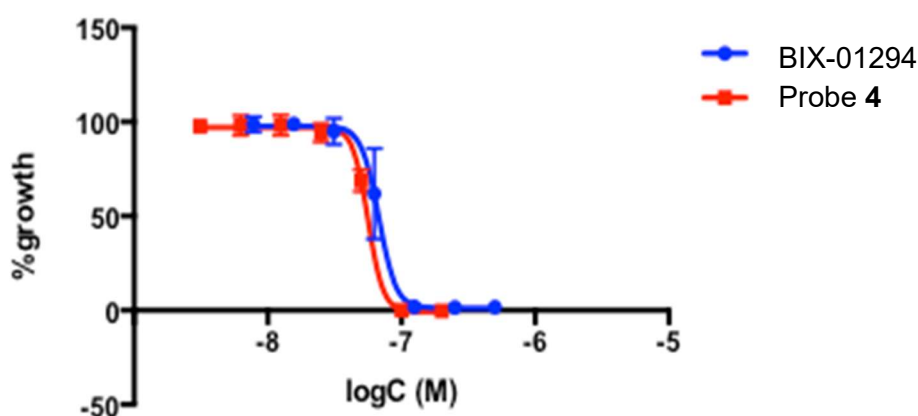


Figure 49: Dose-response curve for Probe 4 compared with BIX-01294 in 3-day SYBR Green I based assay.

4.2.3. Cell Culture and Lysate Preparation

Performed by the Baum Lab at Imperial College London.

P. falciparum 3D7 cultures were maintained at ~2% haematocrit in RPMI-1640-HEPES medium with 0.5% Albumax II, 0.25% sodium bicarbonate, 50 mg L⁻¹ hypoxanthine and 25 mg L⁻¹ gentamicin. Parasites were incubated at 37 °C in an atmosphere of 5% oxygen, 5%

carbon dioxide and 90% nitrogen. Parasites were harvested at 30-40 hours post invasion (trophozoite/schizont stage) at ~6-8% parasitaemia.²¹⁸ For saponin lysis, cells were first spun down (800 g, 5 min) and the supernatant removed. Cells were lysed with saponin lysis buffer I (0.075% saponin in PBS) at rt for 10 min, and then pelleted (2800 g, 4 °C, 10 min). The supernatant was removed, and the pellet washed three times in cold PBS (2800 g, 4 °C, 10 min). The pellets were stored at -80 °C.

Parasites were lysed by nitrogen cavitation.¹⁸⁵ Saponin pellets were thawed on ice, and then suspended in an equal volume of cold PBS with EDTA-free protease inhibitors (Roche cOmplete, 1 tablet in 40 mL PBS).¹⁸⁵ The suspension was added to a Parr 439 45 mL cell disruption vessel at 4 °C, the chamber sealed and a nitrogen pressure of 1500 psi maintained for 1 h. The pressure was released, the suspension centrifuged (10,000 g, 4 °C, 10 min) and the lysate-containing supernatant collected. The protein concentration of the lysate was measured using a NanoDrop (Thermo Fisher), diluted with cold PBS to 3 mg mL⁻¹ (or desired concentration) and stored at -80 °C.

4.2.4. Nuclear Extract Preparation

Performed by the Scherf Lab at the Pasteur Institute, Paris.

P. falciparum 3D7 cultures were maintained at ~2% haematocrit in RPMI-1640 medium with 0.5% Albumax II. Parasites were incubated at 37 °C in an atmosphere of 5% oxygen, 3% carbon dioxide and 92% nitrogen. Parasites were harvested at 30-40 hours post invasion (trophozoite/schizont stage) at ~5-8% parasitaemia.²¹⁸ For saponin lysis, cells were first spun down (800 g, 5 min), the supernatant removed and the cells washed with RPMI-1640 warmed to 37 °C. Cells were lysed with saponin lysis buffer II (0.15% saponin in RPMI-16) at rt for 10 min and pelleted (3250 g, 6 min). The supernatant was removed, and the pellet washed three times in cold PBS (2400 g, 4 °C, 5 min).

For lysate fractionation,²¹²⁻²¹³ the pellets were re-suspended in cytoplasm lysis buffer containing 10 mM HEPES, 10 mM KCl, 0.65% NP40, 1 mM DTT and EDTA-free protease inhibitors (Roche cOmplete), before they were vortexed and incubated at 0 °C for 30 min. Parasites were transferred to a dounce homogenizer where they were lysed with around 200 strokes (parasites were chilled for 30 sec every 30 strokes). Parasite disruption was confirmed under a microscope, the parasites pelleted (16,000 g, 4 °C, 10 min) and the supernatant collected as the cytoplasmic fraction and stored at -80 °C.

Pellets were washed twice with cold PBS (16,000 g, 4 °C, 10 min) and resuspended in nuclear extraction buffer containing 20 mM HEPES, 600 mM KCl, 2 mM MgCl₂, 25 µM ZnCl₂, 1 mM DTT and EDTA-free protease inhibitors (Roche cOmplete). Parasites were sonicated at 4 °C for 10 min (30 sec on, 30 sec off), and the insoluble material pelleted (16,000 g, 4 °C, 10 min). The supernatant was collected as the nuclear fraction and stored at -80 °C.

4.2.5. Lysate Labelling for In-Gel Fluorescence

For dose-dependent studies, *Pf*3D7 lysate in PBS (100 µL, 3 mg mL⁻¹), was incubated with the desired probe (1-100 µM) at 0 °C for 1 hour. For BIX-01294 competition experiments, lysates were first treated with the desired concentration of BIX-01294 (100-2000 µM) at 0 °C for 30 min before adding the desired probe (10-50 µM) and incubated for an additional 1 h at the same temperature. Competition experiments with other inhibitors were carried out in the same way. For those samples to which no probe was added, DMSO was used as a vehicle. Stock solutions of the probe and BIX-01294 were prepared so that the same amount of DMSO was added in all samples, keeping it always below 2%.

The lysates were then irradiated with UV (365 nm, 0 °C, 20 min) and conjugated to AzT by treatment with 6 µL of a pre-mixed solution containing 1 µL of 10 mM AzT in DMSO (0.1 mM final concentration), 2 µL of 50 mM CuSO₄ in water (1 mM final concentration), 2 µL of 50 mM TCEP in water (1 mM final concentration) and 1 µL of 10 mM TBTA in DMSO

(0.1 mM final concentration). After 1 h shaking at room temperature, 1 μ L of 500 mM ethylenediaminetetraacetic acid (EDTA) in water (5 mM final concentration) was added and the lysates vortexed. To precipitate the proteins, methanol (200 μ L), chloroform (50 μ L), and water (100 μ L) were added, and the mixture vortexed and centrifuged (16,000 g , 10 min). The solvent was removed, and the pellets were washed with methanol (2 x 200 μ L) and stored at -80 °C until analysis.

4.2.6. SDS-PAGE In-Gel Fluorescence

The protein pellets were dissolved in 25 μ L of 2% sodium dodecyl sulfate (SDS) and 10 mM DTT in PBS, and the solution was diluted with PBS (75 μ L) and added to 25 μ L of 4x loading buffer (LDS) and 10 mM DTT. Samples were boiled at 95 °C for 10 min and 30 μ g were loaded into each gel lane of a 4-12% Bis-Tris Nu-PAGE pre-cast gel (Invitrogen) and resolved using gel electrophoresis (SDS-PAGE) in MOPS buffer (Invitrogen) at 160V for 1 h. Gels were fixed in fixing solution for 5 minutes before imaging. Images were acquired using an Amersham Typhoon FLA 7000 fluorescence scanner. Fluorescent images are shown in grey-scale. Gels were stained with Coomassie staining solution for 30 min, and then washed with destaining solution for 1 hour to show total protein loading.

4.2.7. Labelling and Pull Down for Proteomics

Pf3D7 lysate in PBS (200 μ L, 3 mg mL⁻¹) were incubated with probe **2** (10 μ M), probe **4** (50 μ M) or DMSO for control experiments at 0 °C for 1 h, irradiated (365 nm, 0 °C, 20 min) and conjugated to the trifunctional capture reagent AzTB as described above. After 1 h shaking at room temperature, 2 μ L of 500 mM EDTA in water (5 mM final concentration) was added and the lysates vortexed. Proteins were precipitated by adding methanol (400 μ L), chloroform (100 μ L) and water (200 μ L). The mixture was vortexed and centrifuged (16,000 g , 10 min). The solvent was removed, and the pellets washed with methanol (2 x

400 μ L). Pellets were stored at -80 °C overnight and then dissolved in 50 μ L of 2% SDS and 10 mM DTT in PBS, and the solution was diluted with PBS (450 μ L). NeutrAvidin agarose beads (50 μ L) were added to the samples after being washed with 0.2% SDS in PBS (3 x 200 μ L), and the samples were then shaken at room temperature for 90 min. The beads were pelleted (2000 *g*, 2 min) and the supernatant removed. The beads were washed sequentially in 1% SDS in PBS (3 x 400 μ L), 4M urea in PBS (2 x 400 μ L) and 50 mM ammonium bicarbonate (4 x 400 μ L). For each wash step the beads were gently vortexed for 1 min followed by pelleting in a microcentrifuge (2000 *g*, 2 min). The beads were re-suspended with 50 mM ammonium bicarbonate (200 μ L) and 10 μ L of 100 mM DTT was added (5 mM final concentration). The samples were incubated at 55 °C for 30 min with shaking. After centrifugation (2000 *g*, 2 min) and removal of the supernatant, the beads were washed with 50 mM ammonium bicarbonate (200 μ L, 2000 *g*, 2 min) and then re-suspended with 200 μ L of 50 mM ammonium bicarbonate. 15 μ L of 100 mM iodoacetamide (7.5 mM final concentration) was added, and the samples were incubated at room temperature for 30 min in the dark. After centrifugation (2000 *g*, 2 min) and removal of the supernatant, the beads were washed with 50 mM ammonium bicarbonate (200 μ L, 2000 *g*, 2 min), re-suspended with 100 μ L of 50 mM ammonium bicarbonate, and 5 μ L of trypsin in 50 mM ammonium bicarbonate (20 μ g trypsin in 100 μ L of 50 mM ammonium bicarbonate) was added. The samples were incubated at 37 °C overnight with shaking. The beads were pelleted by centrifugation (2000 *g*, 2 min) and the supernatant collected. Beads were washed with 100 μ L of 50 mM ammonium bicarbonate for 10 min, pelleted (2000 *g*, 2 min), and the supernatant collected. Next, beads were washed with 100 μ L of 1.5% TFA for 10 min, precipitated (2000 *g*, 2 min), and the supernatant collected.

For experiments with nuclear extract, the same procedure was followed with the *Pf3D7* nuclear extract diluted to 0.3 mg mL⁻¹ with PBS.

4.2.8. Stage-tip Purification of Peptides

For LC-MS/MS analysis the supernatants containing the peptides were combined and stage-tipped.¹⁹⁵ P200 pipette tips were fitted with 3 layers of SDB-XC extraction disks (Empore®) cut out using an in-house constructed tool. The pipettes were inserted into the hole of a microcentrifuge tube lid. The tips were activated by addition of methanol (150 µL) and the tips centrifuged (2000 g, 2 min). The tip was washed with LC-MS/MS grade water (150 µL). The peptide solution was loaded into the tip and the tip centrifuged again. The water wash was repeated. The peptides were eluted into a clean microcentrifuge tube by addition of 79% acetonitrile in water (2 x 60 µL). The peptides were dried in a Savant SPD1010 SpeedVac® Concentrator (Thermo Scientific) and stored at -80°C until LC-MS/MS analysis. Peptides were reconstituted in 25 µL of 2% acetonitrile in water with 0.5% TFA for LC-MS/MS analysis.

4.2.9. Mass Spectrometry

Performed by the Tate Lab and the Department of Chemistry Mass Spectroscopy Service at Imperial College London.

LC-MS/MS runs were performed on an Easy nLC-1000 system coupled to a QExactive mass spectrometer *via* an easy-spray source (all Thermo Fisher Scientific) as previously described.^{187,194} 3 µL injections of peptide sample were separated on a reverse phase Acclaim PepMap RSLC column (50 cm x 75 µm inner diameter, Thermo Fisher Scientific) across a 2 h acetonitrile gradient containing 0.1% formic acid, using a flow rate of 250 nL min⁻¹. The QExactive was operated in data-dependent mode with survey scans acquired at a resolution of 75,000 at m/z 200 (transient time 256 ms). Up to 10 of the most abundant isotope patterns with a charge of +2 or higher from the survey scan were selected with an isolation window of 3.0 m/z and fragmented by higher-energy collision dissociation (HCD) with normalized collision energies of 25. The maximum ion injection times for the survey

scan and the MS/MS scans (acquired with a resolution of 17 500 at m/z 200) were 20 and 120 ms, respectively. The ion target value for MS was set to 10^6 and for MS/MS to 10^5 , and the intensity threshold was set to 8.3×10^2 .

4.2.10. Mass Spectrometry Data Analysis

The data were processed with MaxQuant version 1.5.7.4, and the peptides were identified from the MS/MS spectra searched against the *Plasmodium falciparum* Swissprot+TrEMBL database (September 2017) using the Andromeda search engine. Cysteine carbamidomethylation was selected as a fixed modification and methionine oxidation as a variable modification. Up to two missed cleavages were allowed. Peptides and proteins were identified utilising a 0.01 false discovery rate (FDR), with “Unique and razor peptides” mode selected (razor peptides are uniquely assigned to protein groups and not to individual proteins). Other parameters were used as pre-set in the software. Data was analysed using Perseus version 1.5.6.0¹⁹⁹ and Microsoft Office Excel 2016. Peptides categorised by MaxQuant as ‘potential contaminants’, ‘only identified by site’ or ‘reverse’ were filtered, and the processed LFQ intensities transformed in $\text{Log}_2(\text{LFQ})$. Two or three valid values were required for identification. Missing values were replaced from a normal distribution with width 0.3 and downshift 1.8. Identified genes were analysed in terms of their viability to disruption by transfection, using the PhenoPlasm database (<http://phenoplasm.org/>).²⁰⁰ For GO term enrichment analysis, the proteins of interest were input into PANTHER (<http://www.pantherdb.org>) to analyse them.^{209-211,219}

4.2.11. Western Blots

Antibodies for PfSET3, PfSET6, PfSET7 and PfSET10 were kindly provided by the Pasteur Institute, Paris.

Pf3D7 lysate in PBS (150 μ L, 3 mg mL⁻¹) was added to 4x SDS loading buffer (50 μ L) and was boiled at 95 °C for 10 min. 30 μ g of protein was loaded into each gel lane before being resolved by gel electrophoresis (SDS-PAGE), using 4-12% Bis-Tris Nu-PAGE pre-cast gels (Invitrogen) for *PfSET6* and *PfSET7* and 3-8% Tris Acetate Nu-PAGE pre-cast gels (Invitrogen) for *PfSET3* and *PfSET10*. Proteins were transferred to PVDF transfer stacks (iBlot 2, ThermoFisher) membranes using a standard 7-minute protocol (ThermoFisher iBlot 2 Dry Blotting System). Membranes were blocked in blocking buffer (0.05% tween20 and 5% BSA in PBS) for 2 h at rt, and then incubated with the relevant antibody in blocking buffer (dilution 1:250) for 16 h at 4 °C. Membranes were washed 3 times with PBST (0.05% tween20 in PBS) before incubation with the secondary antibody (anti-mouse HRP, dilution 1:500) in buffer (10% skim milk in PBST). Membranes were washed 3 times with PBST, before they were developed (ECL Western Blotting Detection Reagents) and imaged for chemiluminescence using the imaging system BioRad ChemiDoc.

4.2.12. Immunoprecipitation of *PfSET1*

Antibodies for PfSET1 were kindly provided by the Pasteur Institute, Paris.

Magnetic Protein A Dynabeads (50 μ L) were washed three times with 200 μ L of washing buffer (PBST with BSA (5 mg mL⁻¹)) using a magnet to pellet the beads. The beads were diluted in washing buffer (200 μ L), before addition of the *PfSET1* antibody (dilution 1:100). The samples were incubated for 1.5 h at rt with shaking, before the supernatant was removed and the beads washed twice with PBST (200 μ L). To crosslink the antibody to the beads, the beads were suspended in 250 μ L of a 5 mM solution of bis(sulfosuccinimidyl) suberate (BS³) in conjugation buffer (20 mM Na₃PO₄, 0.15 M NaCl). The beads were

incubated for 30 min at rt, before the reaction was quenched by the addition of 12.5 μL of 1M Tris HCl (pH 7.5). The beads were incubated for 15 min at rt, and then washed three times with PBST (200 μL).

*Pf*3D7 nuclear extract in PBS (0.3 mg mL^{-1} , 150 μL) was labelled with the probe and AzTB as above. After the click-chemistry was quenched with EDTA, the nuclear extract was added to the Dynabeads, and incubated overnight at 4 °C with rotation. The beads were washed three times with PBST (200 μL) and twice with a cold 100 mM solution of ammonium bicarbonate (200 μL). The beads were suspended in 100 mM ammonium bicarbonate (100 μL), before 10 μL of trypsin solution (2 μg trypsin in 100 mM ammonium bicarbonate) was added. The beads were incubated overnight at 37 °C with shaking. The supernatant was retained, and 7.5 μL of formic acid was added. The peptide-containing supernatant was then stage-tipped and analysed by mass spectrometry as described above.

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