Design, synthesis & evaluation of human Aurora kinase and phosphodiesterase inhibitors
for anti-trypanosomal drug discovery via target repurposing

A dissertation presented
by
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to
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In partial fulfillment of the requirements for the degree of
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ABSTRACT OF DISSERTATION

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Abstract

Neglected tropical diseases (NTDs) represent a group of infectious diseases that blight the lives of approximately one billion people, and collectively cause around 550,000 deaths each year. These diseases are generally concentrated in low-income countries from Africa and Latin America, but are also known to take a heavy toll in parts of South Asia. The current therapies have many limitations such as cost, route of administration, toxicity and the emergence of resistance. The current work is focused on targeting the pathogenic parasite that causes African sleeping sickness, *Trypanosoma brucei* (*T.b.*), by designing new inhibitors by a "target repurposing approach." The strategy implemented in the development of the projects described herein relies on the identification of biological targets in the pathogenic parasite that show homology to biological targets in humans that have already been pursued for drug discovery efforts. This knowledge from these efforts (compounds, structural information) is repurposed in the design, synthesis, and optimization of new agents that inhibit the parasite targets. The current research consists of two distinct projects, focused on the optimization of compounds for two potential drug targets from *T. brucei*: Aurora Kinase 1 (TbAUK1) and phosphodiesterases B1 and B2 (TbrPDEB1 and B2).

*Chapter 1* introduces in detail NTDs and target repurposing as a viable strategy for drug discovery. A comparison of target based screens and phenotype driven screens is also provided. New drug targets such as TbAUK1 and TbrPDEB1/B2 for Human African trypanosomiasis (HAT) are discussed, and their homologous human enzymes are further reviewed.
Chapter 2 describes our first efforts to improve human Aurora (h-Aur) kinase inhibitors for potency against trypanosomes which have led to a preliminary focus on the chemical series related to the Phase II clinical candidate danusertib. This chapter details our results in repurposing the human Aurora kinase inhibitor danusertib, an investigational cancer therapeutic, for treating HAT. New TbAUK1 inhibitors have been designed based on the danusertib chemotype with the guidance of homology modeling of the parasitic enzyme. Some danusertib analogs are effective in parasite killing *in vitro* and display good selectivity over host cells. The concept of ligand efficiency is introduced together with analogs designed to improve it. Synthesis of clickable danusertib analogs to elucidate other off-targets is also reported. AT-9283 is another repurposed human Auk inhibitor that is synthesized and studied as a potential anti-trypanosomal drug. Finally, a possible structure activity relationship cross-over between human Aurora kinase inhibitors is proposed.

Chapter 3 is focused on the synthesis and evaluation of human PDE4 and PDE5 as starting points to develop new anti-trypanosomal drugs. The first part of the chapter studies the tadalafil chemotype, and explains why this scaffold was not pursued further for the design of TbrPDEB1/B2 inhibitors. Then, the next synthetic efforts are revealed with a primary focus on more promising hPDE4 inhibitor chemotypes. The SAR developed on a "Parasite" specific pocket using the human PDE4 inhibitor piclamilast as a starting point is described. Finally, the SAR of another human PDE4 inhibitor GSK256066 is explored in detail and the findings for each explored region are disclosed.
Chapter 4 summarizes the importance of this thesis and provides future directions for the advancement of both projects.
Acknowledgements

The Department of Chemistry & Chemical Biology at Northeastern University is for me a family that I will never forget. During my graduate studies I benefited from the support provided by the Chemistry Department both financially and intellectually. In addition, the relationships that I have built both with faculty members and my fellow graduate students have allowed me to grow not only academically but also as an individual.

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~ Stefan Ochiana
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<th>Description</th>
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<tbody>
<tr>
<td>Ac</td>
<td>acetyl</td>
</tr>
<tr>
<td>Ac$_2$O</td>
<td>acetic anhydride</td>
</tr>
<tr>
<td>AcOH</td>
<td>acetic acid</td>
</tr>
<tr>
<td>ADME</td>
<td>absorption, distributions, metabolism, and excretion</td>
</tr>
<tr>
<td>AMP</td>
<td>adenosine monophosphate</td>
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<tr>
<td>Aur</td>
<td>Aurora</td>
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<tr>
<td>BF</td>
<td>bloodstream Forms</td>
</tr>
<tr>
<td>Bn</td>
<td>benzyl</td>
</tr>
<tr>
<td>Boc</td>
<td>t-Butoxycarbonyl</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>Cdk</td>
<td>cyclin-dependent kinases</td>
</tr>
<tr>
<td>CTB</td>
<td>cell Titre Blue</td>
</tr>
<tr>
<td>cGMP</td>
<td>cyclic guanosine monophosphate</td>
</tr>
<tr>
<td>ClogD</td>
<td>calculated distribution coefficient at pH = 7.4</td>
</tr>
<tr>
<td>ClogP</td>
<td>calculated partition coefficient, lipophilicity</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>CPC</td>
<td>chromosomal passenger complex</td>
</tr>
<tr>
<td>d</td>
<td>doublet</td>
</tr>
<tr>
<td>DALY</td>
<td>disability-adjusted life year</td>
</tr>
<tr>
<td>DBU</td>
<td>1,8-diazabicyclo[5.4.0]undec-7-ene</td>
</tr>
<tr>
<td>DFMO</td>
<td>eflornithine or $\alpha$-difluoromethylornithine</td>
</tr>
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</table>
DIEA  diisopropylethyl amine
DMF  dimethylformamide
DMSO  dimethylsulfoxide
ESI  electrospray ionization
Et  ethyl
Et₂O  diethyl ether
EtOAc  ethyl acetate
GBD  global burden of disease
GMP  guanosine monophosphate
GSK  GlaxoSmithKline
h  hours
HAT  Human African trypanosomiasis
HPLC  High pressure liquid chromatography
h-Auk  human Aurora kinases
HBD  hydrogen bond donors
HTS  high-throughput screening
hu  human
IACUC  Institutional Animal Care and Use Committee
IP  intraperitoneal
IPr  isopropyl
\( J \)  coupling constant
LCMS  liquid chromatography/ mass spectrometry
LDL  low-density lipoprotein
LE        ligand efficiency
LmjPDEB1  Leishmania major PDE
Lys       lysine
m         meta
m         multiplet
MBP       myelin basic protein
MeCN      acetonitrile
MeOH      methanol
Met       methionine
MOLT-4    human acute lymphoblastic leukemia cell line
MMOA      molecular mechanism of action
MPO       multiparameter optimization
M-pocket  metal binding pocket
MW        molecular weight
NMT       myristoyl-CoA: protein N-myristoyltransferase
NMEs      new molecular entities
NTDs      neglected tropical diseases
NMR       nuclear magnetic resonance
ODC       ornithine decarboxylase
P-pocket  parasite pocket
PDB       protein data bank
PDEs      cyclic nucleotide-specific phosphodiesterases
PD        pharmacodynamics
Ph  phenyl
PK  pharmacokinetics
Plk-1  polo-like kinase 1
ppm  parts-per-million
q  quartet
Q-pocket  glutamine and a hydrophobic clamp that promotes nucleotide binding
RNAi  RNA interference
rt  room temperature
s  singlet
S-pocket  solvent exposed pocket
SAR  structure-activity relationship
t  triplet
TBAF  tetrabutylammonium fluoride
TbAUK1  *Trypanosoma brucei* Aurora kinase 1
TbAUK2  *Trypanosoma brucei* Aurora kinase 2
TbAUK3  *Trypanosoma brucei* Aurora kinase 3
*T.b.b.*  *Trypanosoma brucei brucei*
TBDPS  tert-butyldiphenylsilyl
*T.b.g.*  *Trypanosoma brucei gambiense*
TbNMT  *Trypanosoma brucei* N-myristoyltransferase
*T.b.r.*  *Trypanosoma brucei rhodesiense* and *T.b. gambiense*
TbrPDEB1  *Trypanosoma brucei* Phosphodiesterases B1
TbrPDEB2  *Trypanosoma brucei* Phosphodiesterases B2
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>TEA</td>
<td>triethylamine</td>
</tr>
<tr>
<td>THF</td>
<td>tetrahydrofuran</td>
</tr>
<tr>
<td>Thr</td>
<td>Threonine</td>
</tr>
<tr>
<td>TLC</td>
<td>thin-layer chromatography</td>
</tr>
<tr>
<td>TPSA</td>
<td>topological polar surface area</td>
</tr>
<tr>
<td>TPP</td>
<td>target product profile</td>
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Chapter 1: Background

Part A: Neglected tropical diseases and the strategy for anti-trypanosomal drug discovery
1.1 Neglected tropical diseases (NTDs)

1.1.1 Introduction to NTDs

NTDs are mainly represented by a group of chronic illnesses that cause high morbidity and mortality particularly in regions affected by poverty.\textsuperscript{1,2} There are 13 parasitic and bacterial infections that are part of this group: ascariasis, hookworm infection, trichuriasis, lymphatic filariasis, onchocerciasis, dracunculiasis, schistosomiasis, Chagas’ disease, human African trypanosomiasis, leishmaniasis, Buruli ulcer, leprosy, and trachoma.\textsuperscript{1,3,4} Annually, the number of deaths caused by NTDs is over 500,000 and the combined number of disability adjusted life years lost associated with this group has been estimated to be higher than malaria and tuberculosis.\textsuperscript{4,5} The disability-adjusted life year (DALY) is a single health metric that was introduced in 1990 by the authors of Global Burden of Disease (GBD) to facilitate the direct comparison of the burden of various diseases.\textsuperscript{6} This measurement more fully accounts for the health gaps by evaluating both the time lived with disability and the time lost due to early mortality.

Furthermore, the advanced arsenal of technologies that is currently available to our society is unquestionable, yet the gap in health outcomes is still very real.\textsuperscript{7} This phenomenon can be explained in part by the lack of financial incentive for research investment in the private sector; pharmaceutical companies cannot recoup the research and development costs associated with these types of medicinal chemistry programs due to the poor markets represented by NTDs. Nevertheless, over the past decade the translational gap that has been observed in the field of NTDs has begun to close due to a number of public-private collaborations that have emerged.\textsuperscript{7,8}
The focus of this thesis is on a group of diseases among NTDs which belong to the trypanosomatid family of the kinetoplastida order and are mostly found in the poorest areas of the globe. These diseases are regarded as the "most neglected diseases." Specifically, *Trypanosoma* spp. family members cause Chagas disease, which is prevalent in South America, and sleeping sickness which is common in Africa, whereas *Leishmania* spp. is endemic in the Horn of Africa, South Asia, and Latin America (Figure 1.1). In addition, these three neglected

![Figure 1.1](image.png)

tropical diseases have the highest rates of death. The current strategies to keep these diseases under control are focused on surveillance, early identification and treatment, and vector control. However, these tasks are also challenging due to a shortage of appropriate diagnostic tools and safe drugs.

1.1.2 A special focus on African Sleeping Sickness

Human African trypanosomiasis (HAT), more commonly known as sleeping sickness, is caused by two subspecies of Trypanosoma brucei (T.b): T.b. rhodesiense and T.b. gambiense. These two morphologically identical parasites have different locations where they predominate, namely T.b. gambiense (humans are the primary reservoir) is common in west and central African countries whereas T.b. rhodesiense (animals are the primary reservoir) is endemic to eastern and southern African countries. The transmission of these extra-cellular protozoan parasites is achieved via insect vectors (tsetse flies). These two subspecies also present two

distinct clinical stages. The first stage consists of the replication of the trypanosomes in the blood and lymphatic system, then in the next stage the parasites cross the blood-brain barrier and the patient experiences neurological symptoms that get worse with time resulting in somnolence, coma and ultimately death.\textsuperscript{14,15} However, the speeds of morbidity and mortality differ between these subspecies since \textit{T.b.gambiense} produces slow onset chronic trypanosomiasis whereas \textit{T.b.rhodesiense} has a rapid onset which leads to death of around 80\% of patients within half a year.\textsuperscript{14,15} A depiction of the transmission cycle of \textit{T.b. rhodesiense} is also included (Figure 1.2). This illustration shows that the transmission cycle initially takes place in a tsetse-bovid-tsetse cycle, but infrequently the occasional infection of humans takes place leading to HAT.

The current population at risk for HAT is around 60 million (for both forms considered together) and is located in sub-Saharan Africa (Figure 1.3). Another challenge faced with this parasitic disease is that it spreads very quickly when there are no proper surveillance and treatment programs. Despite the fact that the reported incidence of the disease is only around 25,000-50,000 cases per year, the absence of proper surveillance for the afflicted population suggests that a more realistic estimation may be closer to 300,000 cases.\textsuperscript{16} An extreme example may be found in countries struggling with social unrest, where up to 72\% of the rural population was found to be infected with HAT.\textsuperscript{16} Furthermore, the data also suggest that in very remote areas some communities may have been altogether eradicated by HAT.\textsuperscript{16}
1.1.3 Current treatments for HAT

The current drugs used to treat sleeping sickness were developed decades ago, are in short supply, toxic and are also inefficient due to an increase in drug resistance.\textsuperscript{17} There are four licensed drugs for this disease, two for each stage (\textbf{Figure 1.4}).\textsuperscript{18} For stage one of the disease

![Chemical structures of the four drugs available for sleeping sickness treatment.](Image)

\textbf{Figure 1.4.} Chemical structures of the four drugs available for sleeping sickness treatment.

the two drugs are suramin\textsuperscript{19} and pentamidine,\textsuperscript{20} while for stage two (neurological phase) there are melarsoprol\textsuperscript{21} (active against \textit{T. b.gambiense} and \textit{T. b. rhodesiense}) and eflornithine\textsuperscript{22} (effective only for \textit{T. b. gambiense}).

Suramin, developed around 1920, is used only for the first stage of the disease since it cannot permeate the blood-brain barrier. This can be explained by examining its scaffold, namely a polysulphonated naphthyl urea that is negatively charged at physiological pH.\textsuperscript{7} The exact mechanism through which suramin works against sleeping sickness is uncertain, but it is suggested that a possible mechanism of action is the deprivation of the parasite from cholesterol
and phospholipids by reducing LDL uptake. Additionally, this drug must be administered via injection and it is plagued with various side effects such as emesis, fever, mucocutaneous eruptions, polyneuropathy, hematological toxicity and, rarely, renal failure.

Pentamidine was first discovered in the late 1930s, and it is currently produced and donated to the World Health Organization by Sanofi-Aventis. The mechanism of action is still unclear. What is more, there is no clear evidence of resistance to pentamidine, but from an epidemiological standpoint this might be seen as a "case of temporary good luck." Trypanosomes can quickly become resistant through genetic manipulation. Pendamidine is administered by intravenous or intramuscular injection daily for 7 to 10 days. However, its clearance is very slow (half-life is very long- few weeks) and the drug accumulates to high levels during the treatment period.

Melarsoprol was developed in 1947 after around 12,000 arsenical compounds were synthesized and screened for the treatment of sleeping sickness. It is currently the only drug for the treatment of both stage two infections \textit{T. gambiense} and \textit{T. rhodesiense}. The mechanism of action is still unclear and the side effects of the drug are myocardial damage, hypotension, exfoliative dermatitis and reactive encephalitis. This last side effect is observed in 5-10\% of the patients, and the fatality among patients who experience this can be up to 50\%. Finally, there has also been a disturbing increase in the number of treatment failures in recent years in areas like Angola, Democratic Republic of Congo, southern Sudan and Uganda.

The last drug that is available for HAT therapy is eflornithine (\(\alpha\)-difluoromethylornithine), which was introduced in 1981, and, unlike the other three drugs described, the mechanism of action is known. Eflornithine (DFMO) acts as an irreversible inhibitor of ornithine decarboxylase (ODC), and inhibition of this enzyme stops the synthesis of
polyamine, and thus disrupts intracellular polyamine homeostasis that is needed for the cell to survive.\textsuperscript{24,29} DFMO was originally developed as a cancer therapeutic, but showed only poor efficacy in treating malignancies. Similar to the other drugs described above, DFMO has major drawbacks including exorbitant cost, difficult administration (at intervals of 6 h for 14 days given as short infusions) and side effects such as convulsions (7%), gastrointestinal symptoms like nausea, vomiting and diarrhea (10%-39%), bone marrow toxicity leading to anemia, leukopenia and thrombocytopenia (25-50%), hearing impairment (5%) and alopecia (5-10%).\textsuperscript{30}

However, DFMO is the main back up drug for treating \textit{T. gambiae} infected patients who relapse after treatment with melarsoprol.\textsuperscript{30,31} Unfortunately, its use is limited to \textit{T. gambiae} infections and not \textit{T. rhodesiense} which is not sensitive to DFMO, an effect hypothesized to be due to the more rapid regeneration of ODC in \textit{T. rhodesiense}.\textsuperscript{32}

Nevertheless, the future for new drugs for HAT looks indeed promising. For example a new drug candidate SCYX-7158 (\textbf{Figure 1.5}) has emerged from a novel class of boron-containing small molecules and is effective against both subspecies of the parasite \textit{in vitro} as well as \textit{in vivo}.\textsuperscript{33} Most importantly, this new orally-active benzoaborole compound was also effective for the treatment of stage two of HAT. However, the mechanism by which SCYX-7158

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{scyx7158.png}
\caption{SCYX-7158 is a new promising drug for HAT.}
\end{figure}

9
is trypanocidal is not currently known. This new potential drug for HAT is the product of a collaboration of a biotech company SCYNEXIS in Research Triangle Park, North Carolina and Anacor Pharmaceuticals in Palo Alto, California which was sponsored by a non-profit organization known as Drugs for Neglected Diseases initiative, based in Switzerland. This orally available drug for treating stage one and two of HAT was recently advanced in Phase I clinical trials in healthy adults to determine its safety and tolerability.

To sum up, the current therapies are limited by the cost, route of administration, toxicity to the patient, and drug resistant trypanosomes. Since the pipeline for new drugs is so weak, there is an acute need for new lead compounds for HAT.

1.1.4 Target product profile for HAT

A target product profile (TPP) is the enumeration of the necessary attributes that are needed for a specific drug to become a clinically successful medicine and to provide a substantial advantage over current therapeutics. It is used to establish the target patient population, what the adequate levels of efficacy and safety are, the needed dosing route and schedule, the desired properties of the formulated drug, and also the cost associated with making the drug. The compiled TPP involves the accretion of information of what is best for the patient which is provided by health care workers and physicians, health regulators and policy makers typically from the regions where the diseases are endemic. As per the definition of TPP this tool must account first for the existing therapies, and then establish the criteria needed for the advancement of new drugs over the old ones.
The TPP for HAT is shown in Table 1.1. The TPP profile described should be the first measurement of analyzing how feasible a project is, analyzing the progress made and also be used as the guiding steps in any drug discovery program pertaining in this case to HAT. During the earlier phase of any drug discovery process, the TPP can be used to establish the attributes needed for further drug advancement and driving the decision-making process.

Finally, Wyatt et al. note that using TPP guidelines provides the NTD research community a means to conserve resources. If researchers were to follow the TPP guidelines many compound series would not achieve the criteria set forth by TPP, thus never reach the market and the rapid closure of these respective projects could allow the diversion of resources to other more promising areas.
1.2 Drug discovery by target repurposing

1.2.1 Target repurposing in the context of NTDs

Target repurposing is the strategy applied in the development of the projects described in this thesis. This approach relies on the identification of biological targets in the pathogenic parasite that show homology to biological targets in humans that have already been pursued for drug discovery efforts. This knowledge from these efforts (compounds, structural information) is repurposed in the design, synthesis, and optimization of new agents that inhibit the parasite targets. Pollastri and Campbell in their review on the same topic highlight that target repurposing takes advantage of two main assumptions, namely, that many drugs are known to bind specific proteins, and that drug discovery in an industrial setting is protein target focused. The central premise is that evolution has shown similarity between proteins of various organisms, and therefore it is expected that some features will cross over, such as binding and active sites. Therefore, it seems plausible that a protein that is present in both human and parasites can in essence be targeted using a drug that was originally designed for humans. It should be understood that “similarity” between these proteins can vary a lot, and that the targeted enzymes need to be validated as essential for parasite survival.

Target repurposing is possible because the genomes of many pathogens have been sequenced, and now essential parasite targets with human homologs can be readily identified. This information, now available, allows for the prediction and confirmation of the desired parasite protein sequences which can be then compared (sequence identity, similarity etc.) to the
human targets. Then, the next step is to select those parasitic targets that have human homologs with established drug discovery programs. This will provide to any researcher that pursues this strategy an abundance of invaluable lead matter and data (structure, SAR, toxicity) that can be repurposed. Thus, a new drug-discovery program can be initiated that should, at least in theory, provide quickly drug candidates. However, target repurposing does involve the risk that some of these compounds that come from previous programs against human targets may ultimately face the challenge of toxicity via inhibition of the same or similar human targets.\textsuperscript{36}

This concern can be answered by arguing first that selectivity between the parasite and the human targets is achievable. Nonetheless, even if for some reason this was not the case, then taking in consideration the severe pathology of some parasitic diseases and the sub-standard medication available, some off-target effects might be acceptable risks.\textsuperscript{36}

\textbf{1.2.2 Successful programs that have applied the concepts of target repurposing}

The first successful program to be described herein is represented by one of the four drugs currently used to treat HAT, namely eflornithine. This compound which was first recognized for its antiproliferative properties was initially explored for cancer chemotherapy.\textsuperscript{37} The efficacy of this drug for cancer was meager, and thus the clinical pursuit was halted.\textsuperscript{36} Nevertheless, others recognized the potential as a new trypanocidal drug, since it was noted that trypanosomes utilize a homologous ODC enzyme that could be targeted by DFMO.\textsuperscript{38,39}

Another interesting program looked at \textit{myristoyl-CoA: protein N-myristoyltransferase} (NMT). This enzyme catalyzes an important post-translational modification in proteins, namely protein \textit{N}-myristoylation which is the transfer of a molecule of myristic acid to the amino group of \textit{N}-terminal glycine residues.\textsuperscript{36,40} NMT has been looked at as a possible target for cancer
therapeutics and for fungal infections. Thus, a reservoir of inhibitors has been established for this enzyme. As probably anticipated in *T. brucei* there is indeed a homologous enzyme TbNMT which has been validated as essential via RNA interference. What is more, out of the two human isozymes of NMT that share a 77% identity (NMT1 and NMT2), NMT2 is the closest human homologue to TbNMT showing approximately 55% identity and 69% similarity. Therefore, TbNMT could be quickly accessed via a benchmark screening of known human NMT inhibitors. Nonetheless, the Drug Discovery Unit at University of Dundee in Scotland opted to approach the *Tb*NMT target differently by using a high-throughput screen (HTS) of 62,000 compounds. The research team from Dundee discovered compound B (DDD85646) from the initial HTS hit compound A (DDD64588), and this was achieved via extensive SAR studies that involved in house synthesis of 120 compounds and also the purchase of 30 follow up analogs (Figure 1.6). The potency of compounds A and B is also included together with the data against recombinant *Tb*NMT and *hu*NMT, as well as against BF of *T. brucei* and for the proliferation of a prototypical mammalian cell type (MRC5). The chemistry driven optimization has successfully delivered the lead compound B which is a highly potent, trypanocidal, and orally active inhibitor of *Tb*NMT. In addition, compound B was also shown to cure rodents of infection with *T.b. brucei* and *T.b. rhodesiense* strains. However, this current lead has limited brain penetration (brain/blood ration of <0.1) and this seems plausible given its current physiochemical properties (Polar Surface Area (PSA) = 92, MW = 495) and the fact that it is also a weak P-glycoprotein substrate. Finally, the research team at Dundee is presently working to optimize the current lack of selectivity against *hu*NMT (Figure 1.6) and also the inability of this compound to cross the blood-brain barrier.
Overall, this particular case is another promising example that target repurposing is a feasible concept. Though they did not use existing chemical matter discovered for *huNMT*, their program was driven by target knowledge, revealing highly potent inhibitors of *TbNMT* that displayed single digit nanomolar IC$_{50}$ values. However, the TPP for a new drug for HAT requires compounds to be safe and efficacious against both stage one and two of the disease. This optimization must be achieved ideally retaining the excellent potency against *TbNMT* and BF *T. brucei* cells.

**Figure 1.6** Identification of NMT lead compound **B** via extensive SAR.
1.3 Target based screens vs. phenotype driven screens

In the past decades two broad types of screens have had a dominant role in the process of early stages of the development of drugs: phenotypic screens and target based screens. The phenotypic screen studies the effects that drugs have on cells, tissues or whole organisms. On the other hand, the target based screen is focused on measuring the effect that compounds display against a purified target using *in vitro* assays.

1.3.1 Drugs that were discovered through target based screens

The target-based approach has allowed the discovery of 17 of the 50 first in class new molecular entities (NMEs) that were approved between 1999 and 2008 by the US Food and Drug Administration. Using this method, drugs are optimized for potency and physicochemical/metabolic properties, often aided by the use of structural information about the target of interest. Such projects are often initiated by screening of small-molecule libraries to identify initial leads for the desired target. It should be highlighted that knowledge of targets does not mean that the path to drug discovery is an easy journey. As an example, despite the fact that renin has been an established target for the treatment of hypertension for a long time (with a significant amount of available structural biology data), the challenge of developing orally active inhibitors was burdensome. In addition, some drugs (e.g. kinase inhibitors gefitinib, imatinib, HIV integrase inhibitors e.g. raltegravir etc.) that were discovered via target based approaches lacked the proper identification of the molecular mechanism of action (M_MOA) at the target that
was originally chosen for screening. This information underlines that in reality the MMOA at the target is not always clear when starting a drug discovery strategy. The target-based approach is advantageous due to the molecular and chemical knowledge that is readily accessed and used when testing certain hypotheses.

1.3.2 Drugs that were discovered through phenotypic screening

During the time frame analyzed by Swinney and Anthony (1999-2008) there were 28 first-in-class small molecule NMEs which were found via phenotypic screening that either focused on effecting a specific phenotype (25 NMEs) or were identified simply through chance (3 NMEs). The strategies that pursued a particular phenotype relied on assays that provided information about a desired physiological phenomenon (without knowing the MMOA), and many times the newly identified leads were later evaluated for their MMOA. A vast majority of drug discoveries benefitted by employing known chemical classes that were then matched with a specific phenotype (e.g. nucleoside analogues screened as potential anticancer and antiviral agents). In addition, the development of drugs like ezetimibe, linezolid, pemrolast, etc. was achieved via random library screening using a phenotypic output. The main advantage of a phenotypic approach is the fact that assays do not rely on the knowledge of MMOA, and thus it is considered that the therapeutic impact would be better for a specific disease since it probes the full molecular signaling pathway in a way that is both efficient and unbiased versus the target based assays which rely on a predefined and occasionally poorly validated target. One challenge associated with the phenotypic approach is the optimization of
the molecular properties of the drug leads without having available the design parameters that are provided by prior knowledge of the MMOA.47

1.3.3 Comparison of target- and phenotype-based approaches

The target based approach in drug discovery is typically guided by hypothesis, and for a new drug to emerge on the market there are three main hypotheses that must be tested.47 First, one hypothesis must encompass the relevance of clinical activity in patients for a drug candidate that initially showed activity in preclinical screens. Then, the other two hypotheses must establish that the target investigated is relevant in human disease and also that the drug candidates of which the MMOA is known must be able to exert the needed biological response. The investigation of all these hypotheses is time consuming and requires significant resources.

The phenotypic screening approach, where there is a screening assay that correctly evaluates the human disease, is advantageous because there is not set in stone target hypothesis or MMOA. More importantly, the phenotype approach simultaneously optimizes for the desired physiochemical properties and whole cell/organism effects, whereas the target-based approach focuses on single target(s) that may or may not translate to the desired phenotype in cell or organisms.

To sum up, these two strategies are both feasible and can often be combined when at least one MMOA is presumed, thus helping the research teams reach their goals faster. Finally, in Table 1.247 is shown that when analyzing these two discovery strategies the phenotypic approach was favored by central nervous system disorders and infectious diseases, while the target based strategy provided more drugs for cancer, and metabolic diseases.
<table>
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<th>Phenotypic screening</th>
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<tr>
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<td>3</td>
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Chapter 1: Background

Part B: Drug targets for HAT
1.4 Discovery and development of Aurora kinase inhibitors

1.4.1 Human Aurora kinases as drug targets

Human cancers may be caused by abnormalities in DNA sequence. An area of intense research in this field has been identifying components of the mitotic machinery that can be targeted, in order to stop the progression of various tumors. Among these targets that were explored we find critical signaling kinases such as Aurora, Polo-like kinase 1 (Plk-1) and the cyclin-dependent kinases (CDKs).

Aurora kinases represent a major therapeutic target in the cell mitotic pathways. These kinases are a family of three highly homologous serine/threonine protein kinases (Aurora A, B, C) that control cell mitosis. They were first discovered in 1995, and three years later it was observed that Aurora kinases are expressed in human cancer tissue. Therefore, these kinases have been intensively studied by both academia and industrial oncology communities, resulting in over ten Aurora inhibitors that have progressed to clinical assessment.

Aurora A plays a significant role in many of the processes that are vital for mitosis, and the depletion of Aurora A results in major mitotic defects. Briefly, when Aurora A is inhibited the cell profile shows a delay in mitotic entry, then defects in the separation of chromosome due to aberrant spindle formation that ultimately results in non-diploid DNA content. More importantly depletion studies have suggested that, after these mitotic defects, apoptosis will ensue for the cells that were treated with Aurora A inhibitor.

The chromosomal passenger complex (CPC) that is pivotal for both the progression through and completion of mitosis has as its catalytic component Aurora B. Depletion studies
were also performed for Aurora B, resulting in major defects in mitosis and production of polyploid cells.\textsuperscript{18} What is more, treatment of cells with Aurora B inhibitors caused mitotic defects, and as a result the damaged cells were subjected to apoptosis.\textsuperscript{59,60} There has been less research on the role of Aurora C, and the importance of this enzyme in mitosis is not well established.\textsuperscript{61} However, based on the available data it looks like the role of Aurora C might overlap with that of Aurora B, perhaps suggesting some degree of redundancy between the two enzymes.\textsuperscript{62}

1.4.2 \textit{Trypanosoma brucei} Aurora kinase 1 (TbAUK1) and h-Auk inhibitors

A search of the Trypanosome Genomic Data Base identified three Aurora kinase homologues that were assigned as TbAUK1, 2 and 3.\textsuperscript{63} These parasitic enzymes display a 30-40\% sequence identity and 50-60\% sequence similarity with the human Aurora kinases, with the notable exception of TbAUK3 that has a longer C terminus sequence (Figure 1.7).\textsuperscript{63} In addition, these three homologues have similarities with the human Aurora kinases when one compares the activation loop of the homologues with the catalytic domain of the Aurora kinases, and the homologues show the D (destruction)-box near the C terminus that is also present in all Aurora kinases.\textsuperscript{63} Tu \textit{et al.} also mention that the A-box domain, which is characteristic for human Aurora A, is not present in the three trypanosome kinases.\textsuperscript{63} This knowledge and an alignment of the protein sequences indicate that TbAUK1, 2 and 3 are close homologues of human Aurora B kinase (Figure 1.7).
These three parasitic enzymes were knocked down individually via RNA interference, and only the knockdown of TbAUK1 was determined to be critical in cell cycle regulation. The TbAUK1 was also validated as essential for infection in a mammalian host and this was done by using mice that were inoculated with BF TbAUK1 RNAi cells. Briefly, the control mice without induced TbAUK1 RNAi showed high levels of infection after 3 days \(1 \times 10^8\) trypanosomes per ml, and died by day 4 and 5 whereas RNAi knockdown of parasites led to mouse infections below detectable levels of parasitemia. Overall, this information shows that TbAUK1 is critical for infection in mice, and also that this parasitic enzyme is needed for cell

Figure 1.7 Structures of the three Aurora homologues from *T. brucei* and mammals. Reprinted with permission from Tu, X.; Kumar, P.; Li, Z.; Wang, C. C., An aurora kinase homologue is involved in regulating both mitosis and cytokinesis in Trypanosoma brucei. *J Biol Chem.* 2006, 281 (14), 9677-87. Copyright (2006) by the American Society for Biochemistry and Molecular Biology.
cycle progression. TbAUK1 was determined to be responsible for controlling mitosis, kinetoplast replication and also the initiation of cytokinesis. Our collaborators have produced compelling preliminary data showing the importance of TbAUK1 for infection. For example, established human Aurora inhibitors (Hesperadin, VX-680, danusertib, Figure 1.8) were shown to inhibit TbAUK1 activity to various levels. We have observed that these inhibitors and MLN8237, PHA-680632 and AT-9283 (Figure 1.8) block cell cycle progression in cultured bloodstream forms. Notably, published results suggest that drugs like Hesperadin and VX-680 lead to a phenocopy of RNAi suppression of TbAUK1 expression.

The validation of TbAUK1 as a drug target is just the first step in a long process of designing drugs that can be taken to clinical trials. This thesis describes our efforts on trying to speed up this process by repurposing ATP-competitive compounds that are already in different pre-clinical or clinical stages for human Aurora kinases. There is a rich reservoir of compounds available for this purpose since Aurora A and B represent important targets for treating human cancers. Our initial synthetic efforts on this project were focused on repurposing danusertib an established human Aurora kinase inhibitor that we have also observed to be an inhibitor of TbAUK1 and effective in parasite killing in vitro. This work is described in Chapter 2.
Figure 1.8 Benchmark screening results of human Aurora kinase inhibitors
1.5 Phosphodiesterase inhibitors

1.5.1 Human phosphodiesterase inhibitors (PDE)

The PDE superfamily is composed of 11 gene families that are highly related and linked structurally, and also incorporates over 60 distinct isoforms. Each PDE family has between one to four genes, and many of these genes are responsible for the generation of multiple isoforms. This superfamily of mammalian cyclic nucleotide phosphodiesterases preferentially degrade the nucleotide 3',5'-cyclic phosphates cyclic guanosine monophosphate (cGMP) and cyclic adenosine monophosphate (cAMP) to the hydrolysis products 5'-GMP and 5'-AMP. Both cAMP and cGMP are critical intracellular second messengers that play a role in the transduction of a varied group of growth factors and physiologic stimuli. Interestingly, some PDE family members hydrolyze solely cAMP (PDE4, PDE7 and PDE8), or cGMP (PDE5, PDE6 and PDE9) whereas others affect both cAMP and cGMP (PDE1, PDE2, PDE3, PDE10 and PDE11). Besides differing substrate specificity, the PDE family members vary in the levels of distribution of tissue, the specificities of inhibitors, and also in mode of regulation.

In addition, some universal features observed for the PDE families are a highly conserved catalytic core, a paired regulatory region, and also a distinctive amino-terminal region that is responsible for isoform specificity. The catalytic core (270 amino acids) displays high sequence similarity between members of the same PDE gene family (>80%) whereas this similarity decreases when other PDE gene families are compared (25-40% identities). Furthermore, the catalytic core has a histidine-rich PDE signature sequence motif and a binuclear metal ion center consisting of a zinc ion (Zn\(^{2+}\)) and most probably a magnesium ion (Mg\(^{2+}\)).
addition, the three-dimensional structures of the catalytic domains have been reported for PDE1B, PDE2A, PDE3B, PDE4B/4D, PDE5A, PDE7A, PDE9A and PDE10A2. The information from the high-resolution co-crystal structure of PDE4B/D, PDE5A and PDE1B has shown an invariant glutamine (conserved across all PDEs) that is responsible for substrate specificity in an orientation specific fashion. However, for PDE10A2 where the invariant glutamine is locked by two hydrogen bonds, this idea of "glutamine switch" for substrate specificity is not supported. Moreover, the structure-based sequence alignment at the substrate (cAMP and/or cGMP) binding pocket presents impressive variation of amino acids between PDE families (PDE1 -PDE11) and thus suggests that this variation is the main determinant of both size and shape of the pocket, and ultimately for substrate specificity.

The PDE4 and PDE5 enzymes are a major focus of this thesis, and warrant a closer study. To begin with, the catalytic domain of PDE4 shows a compact alpha helical structure that is composed of 16 helices which are separated in three subdomains. The catalytic site, which is highly conserved among the PDE 4 family, is composed of a metal binding pocket (M-pocket), a side pocket that is solvent filled (S-pocket), and a pocket that has the purine selective glutamine and a hydrophobic clamp that promotes nucleotide binding (Q-pocket). All these regions are shown in Figure 1.9. The overall topology of PDE5 is rather similar to the homologous enzyme PDE4, but the sequence identity between the two enzymes in the catalytic region is only 23%. The PDE5 active site is also divided in the same three pockets just described for PDE4.

Generally, while these pockets are similar there are important differences at the structure level; for example the entry to the PDE5 active site is narrower when compared with the large opening observed in the catalytic pocket of PDE4.
All this information has been used to develop potent and selective PDE inhibitors for various medical needs (e.g. treatment of erectile dysfunction and pulmonary hypertension (PDE5), chronic obstructive pulmonary disease (PDE4)). The success of these efforts is evinced in the approval of various selective PDE inhibitors for clinical use such as PDE5 inhibitors (tadalafil, vardenafil, sildenafil), PDE4 inhibitor (roflumilast) and PDE3 inhibitors.

**Figure 1.9** A) PDEs active site and the three pockets are shown: the metal binding pocket (M) shown in blue, the purine-selective glutamine and hydrophobic clamp pocket (Q) shown in red (divided in 2 subpockets Q1 and Q2) and the solvent filled side pocket shown in green. The compound shown as a stick model in the active site of PDE4B is cilomilast (human PDE4 inhibitor). B) A different view of the PDE active site with bound cilomilast highlighting the S pocket. Reprinted from Structure, 12/12, Card, G. L.; England, B. P.; Suzuki, Y.; Fong, D.; Powell, B.; Lee, B.; Luu, C.; Tabrizizad, M.; Gillette, S.; Ibrahim, P. N.; Artis, D. R.; Bollag, G.; Milburn, M. V.; Kim, S. H.; Schlessinger, J.; Zhang, K. Y., Structural basis for the activity of drugs that inhibit phosphodiesterases, 2004, 2233-47, Copyright (2004), with permission from Elsevier.
(cilostazol\textsuperscript{79} and milrinone\textsuperscript{80}). Additionally, the favorable outcome of all these drugs has attracted a lot of research towards PDEs as drug discovery targets.

All the PDEs are present to some extent in the central nervous system (CNS), and this further makes this gene family a luring source for the development of new drugs targeting PDEs in the CNS.\textsuperscript{81} Indeed, the plethora of knowledge available on phosphodiesterases lends credence to the feasibility of targeting PDEs with molecules that are very potent, selective, safe and with good drug-like properties for oral dosing and CNS penetration.

### 1.5.2 *Trypanosoma brucei* Phosphodiesterases B1 and B2 and PDE inhibitors

The cyclic nucleotide-specific phosphodiesterases (PDEs) constitute another viable class of new drug targets for trypanosomiasis. The genome of *Trypanosoma brucei* is known to code for five different PDEs, and two of these (TbrPDEB1 and TbrPDEB2) are closely related.\textsuperscript{82} The overall sequence identity between these two characterized TbrPDEB genes is approximately 30\% in the N-terminal region, but this percentage increases to 88.5\% all through the remainder of the polypeptides.\textsuperscript{82} Additionally, TbrPDEB1 and TbrPDEB2 are known to code for analogous cAMP-specific PDEs.\textsuperscript{83} The localization of these two enzymes within the parasite is also distinct since TbrPDEB1 is located exclusively in the paraflagellar rod (PFR) of the flagellum whereas TbrPDEB2 is mostly present in the cytoplasm.\textsuperscript{84} Furthermore, RNAi against TbrPDEB1 and TbrPDE2 was shown to be lethal for bloodstream forms of *T. brucei*.\textsuperscript{82} The two enzymes must be both knocked down, as knockdown of only one is not sufficient, thus suggesting that the two enzymes may have compensatory mechanisms one for the other. The fact that the cultured bloodstream form trypanosomes are responsive to the inactivation of the two enzymes emphasize
their potential as targets for trypanocidal PDE inhibitors, hence indicating that the ablation of TbrPDEB1 and B2 is enough to eliminate the parasite. Finally, Oberholzer et al. highlight that their work undoubtedly validated the two parasitic enzymes as viable drug targets and that the development of TbrPDEB inhibitors is the next logical step.

To validate TbrPDEB1 as a target for medicinal chemistry our lab benchmarked an extended collection of known hPDE inhibitors that were either synthesized, received as gifts or purchased (Figure 1.10). The plan was to launch an optimization project based on best or promising hits, and to achieve two main goals: tool compounds useful for validation of TbrPDEs as a therapeutic target and to advance lead compounds that meet preclinical criteria for development. The initial criteria for advancement of advanced lead compounds were: IC$_{50}$< 100 nM, > 100x selectivity over L6 cells, >100x selectivity over human PDEs and other targets, ADME (absorption, distributions, metabolism, excretion)/PK (pharmacokinetics) properties appropriate for oral dosing, solubility >25µg/mL, understanding of PK/PD (pharmacodynamics) relationship and preclinical toxicology. The benchmark screening of hPDE inhibitors has uncovered some possible lead series (Table 1.3). For example, one hPDE4 inhibitor, piclamilast has emerged as a lead compound that showed low micromolar activity (TbrPDEB1 IC$_{50}$: 4.7 µM) against the parasitic enzyme TbrPDEB1.

The preliminary data confirms that trypanosomal PDEs represent a promising target family for an anti-parasitic approach. This thesis describes in detail three hPDE inhibitors that were selected for further SAR (Chapter 3).
Figure 1.10 Structures of the benchmarked human PDE inhibitors reported in Bland et al. *J. Med. Chem.* 2011, 54 (23), 8188-94, and in Table 1.3 below.
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<th>hPDE inhibitor</th>
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1.6 Summary

HAT is one of the many NTDs that is in dire need for better therapeutics. Current treatments are limited by cost, route of administration, toxicity and more recently the emergence of resistance. Identification and validation of the essential enzymes from *T. brucei* TbAUK1 and TbrPDEB1 and B2 has created a potential niche for new trypanosomal drugs that could be designed via a target repurposing approach. Data have been obtained for initial chemical series that have launched the drug optimization projects that comprise this thesis.
Chapter 2: Design, synthesis and evaluation of Auk inhibitors for TbAUK1
2.1 Introduction

As previously described in Chapter 1 of this thesis the drug discovery programs can develop from either target based screens or phenotype driven screens. For the TbAUK1 project our drug repurposing program is a combination of these two approaches. Others\textsuperscript{86,65} were able to validate the TbAUK1 via small molecule inhibitors. However, bearing in mind that 70\% of first-in-class anti infective agents that were discovered between 1999-2008 resulted from phenotypic approaches\textsuperscript{47}, we decided to first pursue a phenotypic screen, informed by the knowledge that TbAUK1 was a validated target that is sensitive to human Aurora inhibitors. Our decision was also influenced by the fact that phenotype based optimization would be expected to be more rewarding in studying pathogens of which the biological pathways are not fully understood. Thus, once potent leads are developed all the possible MMOAs could be also elucidated.

The first steps of the project involved screening inhibitors of the trypanosomal cell cycle, and our starting point was a collection of mammalian Aurora inhibitors that had a large pool of medicinal chemistry and biological data. Two promising drugs that were validated against TbAUK1,\textsuperscript{86,65} namely hesperadin and VX-680, were not pursued because the first has never made it to human clinical trials, whereas the second has been halted in phase II clinical trials. Therefore, we focused our energy towards some other chemotypes that are still in clinical development.
2.2 Initial focus: danusertib, a human Aurora inhibitor

The first chemotype that we selected for evaluation was the pyrrolopyrazole danusertib (1, formerly PHA-739358)\textsuperscript{87} and its predecessor analog PHA-680632 (2, Figure 2.1).\textsuperscript{88}

![Figure 2.1 Pyrazolopyrazole inhibitors of h-Auk.](image)

This compound class is of interest to us since danusertib is well advanced into clinical trials, the chemistry is parallel-synthesis enabled and there is a pool of established medicinal chemistry and structural biology data. A rapid SAR generation seemed feasible, and as such we first

![Figure 2.2 Inhibition of kinase activity by compounds in the inhibitor set.](image)
*Biological assay courtesy of Vidya Pandarinath and Dr. Larry Ruben. Southern Methodist University.*
synthesized three initial analogs to study the effect of simple replacements for the diethylphenyl urea headgroup of 2. Compounds 1, 2, and 5a were selected to be tested against TbAUK1. These compounds were screened at 500 nM in an *in vitro* kinase assay. Our collaborators in the laboratory of Dr Larry Ruben (Southern Methodist University) used AU1-tagged TbAUK1, immunoprecipitated from trypanosome homogenates to test for activity against TbAUK1 because any attempt to generate catalytically active recombinant TbAUK1 failed. The Ruben team has demonstrated using this method that hesperadin inhibits TbAUK1 at 200 nM to the level of a background kinase. Therefore, in a similar manner we decided to establish that our lead compounds 1 and 2 were indeed able to lower kinase activity via direct comparison with hesperadin ([Figure 2.2](#)). In brief, the experiment performed involved pulling down of AU1-tagged kinase with anti-AU1 Sepharose and this was utilized to phosphorylate myelin basic protein (MBP). In [Figure 2.2](#) the top panel shows an autoradiogram whereas the bottom panel displays a Coomassie stain in order to demonstrate that each lane was loaded with an equal amount of MBP. Hesperadin (used as a control to show kinase activity in the pull down assay), compounds 1 and 2 inhibited TbAUK1 similarly, whereas compound 5a (Appendix 1) did not.

The next step was to determine for the compounds shown in [Table 2.1](#) the growth inhibition of *T. brucei brucei* bloodstream form (BF) trypanosomes (90-13 strain) with the Cell Titer Blue® end point assay. Compounds 1 and 2 not only inhibit the activity of TbAUK1 at 500 nM but also cell growth with an effective concentration that inhibits cellular growth by 50% (EC₅₀) in a similar concentration range. In addition, 5a did not show any considerable effect on cell growth whereas the other two analogs 5b and 5c (Appendix 1) displayed similar activity to compound 2. Thus, growth inhibition correlates well with kinase inhibition. All the subsequent analogs were assessed via growth inhibition assays. Since we were interested in assessing the
selectivity of our compounds for trypanosome growth we tested each compound for inhibitory
effect of the acute myelogenous leukemia cell line MOLT-4.89 This particular cell line was of
interest since it overexpresses Aurora kinases A and B when comparing with uninduced
peripheral blood mononuclear cells, and Aurora kinase inhibition leads to selective growth
inhibition of MOLT-4.90 Another feature that made this cell line attractive was that analogous to
trypanosomes, it has the ability to grow in suspension culture and circulate in blood and lymph
fluid.

As it can be seen from Table 2.1 the MOLT-4 cell line growth was blocked by all the

<table>
<thead>
<tr>
<th>Compounds</th>
<th>T. b. brucei EC₅₀ (µM)</th>
<th>MOLT-4 EC₅₀ (µM)</th>
<th>Selectivity MOLT-4/Tbb</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.6</td>
<td>0.15</td>
<td>0.25</td>
</tr>
<tr>
<td>2</td>
<td>4.0</td>
<td>0.22</td>
<td>0.06</td>
</tr>
<tr>
<td>5a</td>
<td>11.1</td>
<td>0.54</td>
<td>0.05</td>
</tr>
<tr>
<td>5b</td>
<td>6.1</td>
<td>3.55</td>
<td>0.58</td>
</tr>
<tr>
<td>5c</td>
<td>5.7</td>
<td>0.94</td>
<td>0.16</td>
</tr>
</tbody>
</table>

\(^a\) \text{T.b.b. Lister 427 90-13.} \(^b\) \text{MOLT-4 acute myelogenous leukemia cell line.} \(^*\) \text{Screening data}
courtesy of Vidya Pandarinath and Dr. Larry Ruben. Southern Methodist University.

initial compounds tested. The selectivity data observed for \text{T.b. brucei} and MOLT-4 are
indicative of (a) differing permeability profiles into parasite cells vs. MOLT-4 cells, and/or (b)
differing structural features that exist in the parasite target enzymes and human cells. This is
critical since the repurposing strategy that we use cannot be successful if selectivity for the
parasitic cells cannot be achieved. The preliminary data suggest that we can indeed repurpose
human Aurora kinase inhibitors by modifying known scaffolds to achieve our goals towards potent and selective drugs of trypanosome growth.

### 2.3 Design, synthesis and evaluation of generation 2 of danusertib analogs

#### 2.3.1 Design of the first library

To further the design of new danusertib analogs and to better understand the initial screening data Dr. Zhouxi Wang developed a homology model of TbAUK1 that was based on the human \(^88\) and mouse \(^91\) Aurora A crystal structures (PDB ID 2BMC and 3D14). A direct comparison of TbAUK1 model (Figure 2.3, Right image) and the published Aurora A/danusertib complex (Figure 2.3, Left image) reveal a similar binding pose. Thus, important ligand-protein

![Figure 2.3](image)

**Figure 2.3** Left image shows the human Aurora A/danusertib complex (PDB ID: 2J50, danusertib (colored in green)); Right image shows the predicted conformation of danusertib docked in the TbAUK1 model. *Docking work courtesy of Dr. Zhouxi (Josie) Wang.*
interactions do not change such as the pyrazinylphenyl tail which is positioned towards the solvent, and also the main H-bonding interactions of the main scaffold (pyrazolopyrrole) with the kinase hinge region. A schematic depiction of danusertib in the kinase ATP binding pocket is shown in Figure 2.4.

![Schematic depiction of regions of interest for 1.](image)

Figure 2.4 Schematic depiction of regions of interest for 1.

We did notice, however, that the head group region of danusertib assumes a flipped orientation in the TbAUK1 binding site, and thus placing the phenyl group into a hydrophobic pocket (Figure 2.3, Right image). We hypothesize that this flip is most likely driven by Met113 in TbAUK1 which is a bulkier hydrophobic amino acid than the smaller polar, Thr217 in human Aurora A.

Additionally, it was observed for human Aurora A that a mutation of T217 to glutamate does confer resistance to other Aurora A inhibitors MLN8054 and MLN8237\(^\text{92}\), and so we considered that this amino acid difference could indeed facilitate the design of selective TbAUK1 inhibitors. This analysis made us cognizant of the geometric difference between 1 and 2. Compound 2 that has a planar urea moiety is not able to accommodate as well this amino acid
change (Thr-Met) in the protein, and this is supported by the decrease in potency against *T. brucei* cultures concomitantly with the decrease in selectivity over MOLT-4 cells (Table 2.1). This observation was further supported by docking studies from where it can be seen that the head group region of 2 is firmly held against Met 113 (Figure 2.5), and this appears to be the main reason why we notice a decrease in activity against the parasitic enzyme. We hypothesized that the tetrahedral geometry of the carbon adjacent to the carbonyl group would accommodate the Met residue and allow the desired headgroup flip of the analogs, and also that lipophilic side chains would potentially improve potency/selectivity since they would fit better in the neighboring lipophilic pocket. With these design principles in mind, we pursued the design of a library.

The first step was the enumeration of a virtual library using 208 arylacetic acids that were commercially available in pre-weighed quantities from ASDI, Inc. Then, this virtual library of compounds was filtered to retain only molecules that had a molecular weight of <500 and cLogP ≤5.0. A diverse subset of 50 analogs was docked into TbAUK1 homology model and, based on |

**Figure 2.5** The predicted conformation of 2 (colored in purple), 8 (colored in yellow), and danusertib (colored in green), docked into human Aurora A (Left image) and in the TbAUK1 model (Right image). The colors in the Right image are shown for sidechain heteroatoms in Lys58 (blue) and Met113 (yellow). *Docking work courtesy of Dr. Zhouxi (Josie) Wang.*
the docking score, a list of 20 compounds was prioritized for library synthesis. The compounds that displayed >40% growth inhibition of *T. b. rhodesiense* at 1 µM would be further progressed into dose-response assays.

### 2.3.2 Synthesis and evaluation of the danusertib analogs

The library synthesis was pursued as shown in Scheme 2.1. The synthesis of the original analogs was also aimed at optimizing the parallel chemistry methodology, besides exploring the SAR on the pyrrolidine. The carboxylic acid intermediates were first converted to the acid chlorides using oxalyl chloride, followed by N-acylation and deprotection of the ethyl carbamate group to give the desired final compounds (Scheme 2.1).

**Scheme 2.1** Synthesis of arylacetamide derivatives 8-18. Reagents and conditions: (a) oxalyl chloride, CH₂Cl₂, DMF. (b) 3, DMF, DIEA. (c)10% Et₃N, MeOH.
We were able to successfully synthesize 19 of the 20 prioritized library compounds, and these analogs were tested at 1 and 10 µM against *T. brucei brucei* BF cell lines (AnTat1.1A, *Table 2.2*). Furthermore, dose-response analysis against the human infective *T. b. rhodesiense*, and MOLT-4 cells (*Table 2.3*) was performed for the compounds that displayed a percent inhibition higher than 60% at 1 µM (8-18). The screening data did not provide compounds of improved potency over danusertib, but an improved selectivity ratio was observed. The most selective compound (8) displayed an approximately 23-fold selectivity, whereas eight other analogs displayed some degree of selectivity.

Compound 8 was found to be most selective, an analog with a napthyl in the head group region, and as we anticipated is predicted to have a flipped conformation and to extend a little deeper in the lipophilic pocket (*Figure 2.5*, Right image). Furthermore, as can be seen from the docked pose of 8, the napthyl head group displays lipophilic interactions with the top of the binding pocket, and here it could also favor a possible π-cation interaction with Lys58 of TbAUUK1 (*Figure 2.5*, Right image). On the other hand, when compound 8 is docked in the human Aurora A (*Figure 2.5*, Left image) an unfavorable steric interaction of the napthyl head group with the pocket is observed, and this is confirmed by the reduced docking score (-6.469) compared to 1 (-10.29) and these tabulated data are shown in *Appendix 3*.

The homology model proved to be a valuable tool that allowed us to interpret the current results, and to also facilitate the biasing of the compounds selected for library design, as we observed that the rank-ordering of our compounds in the docking experiments correlated with the potency observed against the trypanosome cells.
Table 2.2  Single concentration data for danusertib analogs against bloodstream form *T. brucei brucei* (AnTat1.1) cells.

<table>
<thead>
<tr>
<th>Compound</th>
<th>R₁</th>
<th>R₂</th>
<th>% inhib 1 µM</th>
<th>% inhib 10 µM</th>
<th>Std dev 1 µM</th>
<th>Std dev 10 µM</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>H</td>
<td>1-napthyl</td>
<td>82</td>
<td>98</td>
<td>6.8</td>
<td>0.4</td>
</tr>
<tr>
<td>11</td>
<td>H</td>
<td>3-Cl-Ph</td>
<td>35</td>
<td>98</td>
<td>9.2</td>
<td>0.2</td>
</tr>
<tr>
<td>19</td>
<td>H</td>
<td>2,4-di-F-Ph</td>
<td>6</td>
<td>98</td>
<td>3.1</td>
<td>0.3</td>
</tr>
<tr>
<td>20</td>
<td>H</td>
<td>2,5-di-F-Ph</td>
<td>6</td>
<td>98</td>
<td>3.4</td>
<td>0.0</td>
</tr>
<tr>
<td>21</td>
<td>H</td>
<td>2,6-di-F-Ph</td>
<td>20</td>
<td>97</td>
<td>16.4</td>
<td>0.2</td>
</tr>
<tr>
<td>22</td>
<td>H</td>
<td>3,4-di-F-Ph</td>
<td>18</td>
<td>98</td>
<td>9.4</td>
<td>0.1</td>
</tr>
<tr>
<td>18</td>
<td>H</td>
<td>3,5-di-F-Ph</td>
<td>98</td>
<td>98</td>
<td>0.2</td>
<td>0.3</td>
</tr>
<tr>
<td>15</td>
<td>H</td>
<td>2,5-di-Me-Ph</td>
<td>34</td>
<td>99</td>
<td>7.1</td>
<td>0.3</td>
</tr>
<tr>
<td>12*</td>
<td>iPr</td>
<td>Ph</td>
<td>99</td>
<td>99</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>9</td>
<td>H</td>
<td>2,3,6-tri-F-Ph</td>
<td>52</td>
<td>98</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>23</td>
<td>H</td>
<td>2,4,6-di-F-Ph</td>
<td>23</td>
<td>100</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>10*</td>
<td>OMe</td>
<td>Ph</td>
<td>77</td>
<td>97</td>
<td>19</td>
<td>0</td>
</tr>
<tr>
<td>17</td>
<td>H</td>
<td>3-(2-Me-indoyl)</td>
<td>62</td>
<td>98</td>
<td>1</td>
<td>0</td>
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<td>14</td>
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<td>3,5-di-Me-Ph</td>
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<td>99</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>16</td>
<td>H</td>
<td>2,3,5-tri-F-Ph</td>
<td>44</td>
<td>99</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>24*</td>
<td>Me</td>
<td>4-Me-Ph</td>
<td>34</td>
<td>79</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>13*</td>
<td>Me</td>
<td>4-Me-Ph</td>
<td>19</td>
<td>100</td>
<td>1.3</td>
<td>0.8</td>
</tr>
<tr>
<td>25</td>
<td>H</td>
<td>2,4-dimethylthiazole</td>
<td>13</td>
<td>35</td>
<td>6</td>
<td>1</td>
</tr>
<tr>
<td>26</td>
<td>H</td>
<td>2-methylthiazole</td>
<td>23</td>
<td>53</td>
<td>9</td>
<td>5</td>
</tr>
</tbody>
</table>

*Denotes compounds tested as a racemic mixture. Screening data courtesy of Vidya Pandarinath and Dr. Larry Ruben. Southern Methodist University.
A good correlation was observed to the docking scores ($R^2=0.75$ for *T. brucei rhodesiense* and 0.72 for *T. brucei brucei*) when compared with the cellular potency values *(Appendix 3).*66
2.3.3 Discussion of generation 2 danusertib analogs

The results obtained indicate that danusertib, a human Aurora inhibitor, inhibits both TbAUK1 activity and growth of the trypanosome strains, in particular the human infective *T.b. rhodesiense*. The paradigm of drug discovery that we pursued by repurposing an optimized human Aurora inhibitor towards designing drugs for HAT seemed to have provided some very promising initial results. As expected, focused changes to the danusertib chemotype resulted in compounds with decreased activity against mammalian cells, but with retained nanomolar potency against the parasitic cells. Overall, while some analogs of 1 do show loss of potency against trypanosomes, the loss is not commensurate with the one observed for mammalian cells. Therefore, an improved selectivity profile for our anti-trypanosomal compounds was achieved ranging between 2.2 to 23.4 fold. Our analog design was successfully guided by our TbAUK1 homology model, and we anticipated being able to develop other compounds that will display further increases in both potency and selectivity.

In summary, an initial SAR was developed on the danusertib chemotype which led to anti-trypanosomal leads. The activity of these compounds is either in part or perhaps completely driven by inhibition of TbAUK1, an aspect that will be elucidated in the near future. The first library provided us with a better understanding of the danusertib chemotype, and at the same time raised further questions in regards to the headgroup region.

We wished to further explore analogs of 8 and 12 with respect to the limits of the lipophilic pocket surrounding the headgroup region, the impact of alkylation of the α-carbon of the headgroup, and the impact of a chiral center in this region. To answer the new questions we pursued a second library and some very focused singleton analogs.
2.4 Synthesis and biological evaluation of generation 3 danusertib analogs

2.4.1 Synthesis and SAR of generation 3 and singleton analogs

Generation 3 was primarily designed to further explore the potency and selectivity profile observed with the racemic analogs 12 and 13 (Appendix 1). We wished to further explore the impact of alkylation at the \(\alpha\)-carbon of the headgroup. Another question regarding the importance of chirality was supported by the observation that the racemic danusertib 10 showed a lower potency than its R-enantiomer 1 (Table 2.3).

The synthesis of generation 3 of danusertib analogs and other singleton analogs was performed using the general route described in the first part of the chapter (Scheme 2.1). The diverse set of the aryl acetic acids was purchased this time from two other commercial vendors, Sigma Aldrich and Fisher Scientific. The structures of all the analogs that were synthesized as part of generation 3 and singleton analogs are shown in Figure 2.7.

\[\text{Figure 2.6 Design of generation 3.}\]
R₁ =

**A**

27  28  29  30

31  33  34  42

35  37  38  43

36  32

**B**

44  40  41

45  50

*Figure 2.7* A) Generation 3 compounds B) Other danusertib singleton analogs.
Using the screening funnel described previously, the generation 3 analogs were first tested at 1µM and 10 µM for *T. brucei brucei* % inhibition (*Figure 2.8*). The compounds that showed more than 40% inhibition at 1µM were advanced to dose-response analysis. From the initial single point assay results (*Figure 2.8*) we learned that the replacement of the α- hydrogen with the gem-dimethyl (31, 29% inhibition at 1 µM) or spiro compounds (19% inhibition for 34 and 18% inhibition for 42 at 1 µM) had a detrimental effect on activity. The general perception that this head group region is a large hydrophobic pocket that can accommodate steric bulk was further validated (analog 28).

At this point in the project, our biology collaborators decided to switch the cell types from *T. brucei rhodesiense* (YTat1.1) to *T. brucei brucei* (AnTat1.1A). This was done for safety reasons (since YTat1.1 is human infective and requires special handling). In addition, there was a
change in the assay from the original *Cell Titre Blue®* (CTB) to *Resazurin* assay (the descriptions of these two assays is provided in **Appendix 3**). There are a few reasons that the biology team provided for the switch in assays such as: easier handling of the cells, a streamlined assay format, longer incubation times (48 vs. 72 h), which gave a higher signal-to-noise ratio (and therefore increased assay sensitivity).

When the assay format was changed, analogs were tested in both assays for side-by-side comparison (**Table 2.5**). All the compounds described from this point on this project were tested with the new assay. The results of generation 3 library are tabulated in **Table 2.5** and for singleton danusertib analogs are shown **Table 2.4**. The screening data obtained for the new set of analogs highlights that this pocket is indeed sensitive to chirality. The current SAR informed us that the chirality impact on potency is more readily observed with smaller side chains at the α-carbon such as methyl for 43 (S-enantiomer) vs. 38 (R-enantiomer), methoxy 1 (R-enantiomer) vs. 10 (racemic) or 44 (S-enantiomer), than with larger branched alkyls such as isopropyl where no difference between 35 (S-enantiomer) and 36 (R-enantiomer) is observed (**Table 2.4 and 2.5** and **Figure 2.8**). A more detailed study of a larger set of enantiomers is needed to pinpoint if there is indeed a preference of this region for the R enantiomer, as the preliminary data might seem to suggest.

Furthermore, a few other singleton analogs (**Figure 2.7**) were designed which were focused primarily on danusertib and expanding its headgroup further into the pocket by increasing the size of the C-C linker between the α-carbon and the phenyl group from n =0 to n=1 (39, 40) and n=2 (41). In addition compound 45 was designed to probe a possible π-cation interaction with Lys58 of the TbAUK1 by adding an electron donating group (methoxy) on the napthyl head group. Finally, compound 50 design relied on the premise that combining a more
potent analog (12) with a more selective one (8) would create a synergistic effect and perhaps improve the potency and either maintain or increase selectivity. However, the screening data of both analogs 45 and 50 did not further validate our hypothesis (Table 2.5).

Increasing the C-C linker was also detrimental to activity with compounds 39 (9% inhibition at 10 µM), 40 (4% inhibition at 10 µM), and 41 (25% inhibition at 10 µM). This is a strongly unfavorable effect on growth inhibition. This new data, combined with further docking studies of the most potent compounds, will channel the next synthetic steps on this project.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>T.b.brucei % inhibition at 10 µM</th>
</tr>
</thead>
<tbody>
<tr>
<td>39</td>
<td>9</td>
</tr>
<tr>
<td>40</td>
<td>4</td>
</tr>
<tr>
<td>41</td>
<td>25</td>
</tr>
<tr>
<td>44</td>
<td>25</td>
</tr>
</tbody>
</table>

*Screening data courtesy of Vidya Pandarinath and Dr. Larry Ruben. Southern Methodist University.
Table 2.5 Dose-response experiments on the parallel array of analogs of 1 from generation 3 tested against *T. b. brucei* AnTat1.1A strain via two different assays and also in MOLT-4 cells.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>T.b.b.(^b) EC(_{50}) (μM)(^e)</th>
<th>T.b.b.(^c) EC(_{50}) (μM)(^e)</th>
<th>MOLT-4(^d) EC(_{50}) (μM)(^e)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.6</td>
<td>0.33</td>
<td>0.15</td>
</tr>
<tr>
<td>27(^a)</td>
<td>-</td>
<td>1.1</td>
<td>3.85</td>
</tr>
<tr>
<td>28(^a)</td>
<td>0.24</td>
<td>0.64</td>
<td>2.42</td>
</tr>
<tr>
<td>29</td>
<td>0.35</td>
<td>1.05</td>
<td>2.5</td>
</tr>
<tr>
<td>30(^a)</td>
<td>-</td>
<td>1.22</td>
<td>0.54</td>
</tr>
<tr>
<td>31</td>
<td>0.97</td>
<td>1.2</td>
<td>1.3</td>
</tr>
<tr>
<td>32(^a)</td>
<td>-</td>
<td>2.47</td>
<td>2.07</td>
</tr>
<tr>
<td>38</td>
<td>-</td>
<td>0.96</td>
<td>0.42</td>
</tr>
<tr>
<td>36</td>
<td>-</td>
<td>1.85</td>
<td>2.2</td>
</tr>
<tr>
<td>35</td>
<td>-</td>
<td>1.31</td>
<td>2.1</td>
</tr>
<tr>
<td>45</td>
<td>-</td>
<td>1.72</td>
<td>10.8</td>
</tr>
<tr>
<td>50(^a)</td>
<td>-</td>
<td>1.37</td>
<td>7.91</td>
</tr>
<tr>
<td>37</td>
<td>0.32</td>
<td>0.69</td>
<td>0.99</td>
</tr>
</tbody>
</table>

\(^a\) Indicates compounds tested as racemate. \(^b\) *T. brucei brucei* AnTat1.1A strain (CTB assay). \(^c\)*T. brucei brucei* AnTat1.1A strain (Resazurin assay). \(^d\) MOLT-4 acute myelogenous leukemia cell line. \(^*\)Screening data courtesy of Vidya Pandarinath and Dr. Larry Ruben. Southern Methodist University.
2.4.2 Exploring the solvent exposed region- rationale and synthesis

In general, in a medicinal chemistry program different regions of a lead molecule are analyzed independently, and then the best structural combination of fragments are put together in an overarching goal to obtain a potential drug. However, this process has resulted in an increase in molecular size, perhaps improving potency via more extended lipophilic interactions. We would expect, however, that the drug-like properties of these compounds will be adversely affected. To answer this challenge of optimizing potency and keeping the molecular size as low as possible, a straightforward metric has been developed which approximates the binding of free energy per structural element. This metric is known as ligand efficiency (LE) and is generally defined as the free energy of binding divided by the number of heavy atoms (n) in a molecule. This is often approximated by (-Log(IC)50)/n.

Our initial line of thought for reduction of compound size is outlined in Figure 2.9. The ligand efficiency for the proposed analogs is also shown, assuming that no loss of potency is observed. In short, truncation of the solvent exposed region is not expected to adversely impact binding to the enzyme, and so potency was predicted to be the same, though the number of heavy atoms had been reduced. We synthesized two analogs aimed at improving the ligand efficiency, and at initial explorations of the solvent-exposed region of the inhibitor. This was accomplished following Scheme 2.2.
Figure 2.9. Two synthesized analogs and calculated ligand efficiency.
To that end, N-acylation of the free amine of 46a with the desired acid chloride followed by Boc deprotection provided 3a. Final compounds (3b) were prepared from the requisite acid chloride. Unfortunately, compound 48 showed solubility issues, and therefore it was not screened. On the other hand, compound 49 underwent screening and it displayed only 11% growth inhibition at 10 µM. While this could have been due to reduced inhibitory potency at the target, it could also reflect a diminished permeability into the trypanosome cell. More extensive study is needed for this region.

2.4.3 Discussion of other danusertib analogs

This third round of analogs has provided further insights on the danusertib chemotype. The analogs synthesized to explore the α-carbon adjacent to the carbonyl have shown that increasing the alkyl chain does maintain potency of analogs (e.g. 28). In addition, chirality also
plays an important role in the headgroup region as evinced by the preference for the R-
enantiomer (e.g. 1, 38). Interestingly, the hybrid analog 50 and naphthyl analog 45 did not
provide an increase in potency as initially anticipated (Table 2.4). The data from both generation
2 and 3 also seems to suggest that expanding the hydrocarbon chain at the \( \alpha \)-carbon provides
more selective compounds for trypanosomes versus the mammalian cell line MOLT-4.

2.5 What other targets are in play, if any?

2.5.1 Rationale for synthesis of clickable danusertib analogs

As stated in the introductory part of this chapter we acknowledge to have direct TbAUK1
inhibition data only for compounds 1, 2, and 5a, but we were still able to pursue our inhibitor
optimization studies without having conclusive trypanosome kinase inhibition data. Our general
observation is that growth inhibition correlates well with kinase inhibition. However, we
suspected that other off-targets could also be involved (besides TbAUK1 maybe TbAUK3 etc.),
and thus we pursued the synthesis of three tagged danusertib analogs that would potentially
elucidate this question when tested by the biology research team at Southern Methodist
University. Such tagged analogs could be used for affinity chromatography of cell lysates, or by
performing \textit{in situ} labeling of trypanosome enzymes that bind the compounds.

The azide and terminal alkyne functionalities are preferred as tags since they are inert to
most chemical functionalities and show stability to a wide range of solvents, temperature and
pH.\textsuperscript{95} In addition, the use of azides and alkynes and their "click reaction"\textsuperscript{96} for the facile
immobilization of the desired ligands on agarose for affinity chromatography is well established.
Affinity chromatography exploits various interactions between molecules in biological processes, e.g. antibodies and antigens, carbohydrates and lectins, and lastly, but relevant to our work, the interactions between enzymes and inhibitors. On an insoluble support is immobilized one member of the interacting pair and this is used to "fish out" the corresponding agent from a solution that is passed through the column.

2.5.2 Synthesis and evaluation of clickable danusertib analogs

The first synthetic efforts towards the synthesis of the tagged compounds were focused on the synthesis of intermediate 54 via the route described in Scheme 2.3. First, the ester of compound 51a, which was synthesized as previously reported in the literature, underwent hydrolysis to the acid under basic conditions, followed then by the protection of both the acid and the alcohol with the silyl protecting group. Compound 53 was obtained after selective deprotection of the silyl group at the acid functionality using potassium carbonate to give the desired mono-protected product in good yields. Finally, the acid was converted to the acid chloride using oxalyl chloride to give the desired intermediate 54. The synthesis of the desired tagged analogs 58, 60 and 61 was achieved as shown Scheme 2.4. Briefly, compound 46a underwent N-acylation via reaction with intermediate 54, to give compound 55 in good yield. Removal of the Boc protecting group under acidic conditions provided intermediate 56 which underwent N-acylation to give the desired compound 57. The silyl protecting group was then removed using TBAF at room temperature to give the desired compound 59 in very good yields. In addition, in this deprotection reaction the desired first tag compound 58 was isolated as a byproduct.
The alcohol moiety of 59 was converted directly to the azide using diphenylphosphoryl azide\textsuperscript{98} followed by deprotection of the ethyl carbamate to give compound 60. Furthermore, the O-alkylated product 61 was obtained by first reacting the alcohol moiety of 59 with 3-bromoprop-1-yne followed by deprotection of the ethyl carbamate.

Unfortunately, these tags did not show any growth inhibition against the trypanosomes cell cultures: the azide (60) displayed 5\% inhibition at 10 µM, and the alkyne (61) showed 10\% inhibition at 10 µM, rendering these useless for affinity labeling experiments. However, the alcohol tagged compound 58 displayed an EC\textsubscript{50} of 1.78 µM and further studies with this compound are ongoing.
Scheme 2.3 Synthesis of tagged danusertib intermediate 54.
Reagents and conditions: a) NaOH, H₂O:MeOH 4:1, reflux, 3h (100%). b) TBDPSCl, I₂,
imidazole, THF, 48 h (100%). c) K₂CO₃, THF:H₂O 1:1, 50 °C, 2h (56%). d) oxalyl
chloride, CH₂Cl₂, DMF, reflux, 5h (100%).
Scheme 2.4 Synthesis of tagged danusertib analogs 58, 59 and 60.
Reagents and conditions: a) Pyridine, 54, THF, rt, 12h (71%). b) 4M HCl in dioxane, DCM, rt, 24h (100%). c) (R)-2-methoxy-2-phenylacetyl chloride, DMF:DCM 5:1, DIEA rt, 5h (56%). d) TBAF, THF, rt, 1h (79% for 59 and 5% for 58). e) DMF, DBU, diphenyl phosphorazidate, 110°C, 2h (76%). f) 10% Et3N, MeOH, 55°C, 48 h (40%). g) DMF, DBU, 3-bromoprop-1-yne, 110°C, 2h (47%). h) 10% Et3N, MeOH, 60°C, 60 h (50%).
2.6 Other chemotypes explored as trypanosome growth inhibitors.

2.6.1 Design, synthesis and evaluation of AT-9283 and analogs

Another chemotype that we selected for evaluation was a pyrazole-benzimidazole compound represented by AT-9283 (compound 68)\(^99\).

![Pyrazole-benzimidazole inhibitor of h-Auk.](image)

**Figure 2.10** Pyrazole-benzimidazole inhibitor of h-Auk.

Similar to danusertib this compound class is also of interest because AT-9283 is well advanced into clinical trials, the chemistry is parallel-synthesis enabled, and there is a pool of established medicinal chemistry and structural biology data.
Scheme 2.5. Synthesis of AT-9283 and analogs.
Reagents and conditions: a) SOCl₂, THF, DMF, reflux, 2h. b) morpholine, THF, Et₃N, rt, 24h (77%). c) NaBH₄, BF₃·OEt₂, THF, rt, 3h (82%). d) 5% Pd-C, H₂, EtOH, rt, 12h (87%). e) 4-nitro-1H-pyrazole-3-carboxylic acid, EDC, HOBt, DMF, rt, 24h. f) AcOH, reflux, 3h (56%) (g) 5% Pd-C, H₂, DMF, rt, 5h (100%). h) CDI, THF, reflux, 16 h. i) cyclopropylamine, DMF, 100 °C, 12h (59%). j) (R)-2-methoxy-2-phenylacetyl chloride, THF, DIEA, rt, 3h. k) KOH, MeOH, rt, 16h (71%). l) THF, DIEA, ethanesulfonyl chloride, rt, 3h. m) KOH, MeOH, rt, 16h (12%).
Besides synthesizing AT-9283, pyrazole-benzimidazole scaffold (intermediate \textit{66}) was also scaled-up to facilitate the synthesis of libraries. Compounds \textit{68}, \textit{69} and \textit{70} were synthesized as described in Scheme 2.5.

First, the commercially available 3,4-dinitrobenzoic acid (\textit{62}) was treated with thionyl chloride, followed by morpholine to give the amide \textit{63}. Compound \textit{64} was obtained by reduction of the amide with NaBH\textsubscript{4} in the presence of a Lewis acid. Then, the two nitro groups of \textit{64} were reduced to the diamine \textit{65} via hydrogenation under palladium catalysis. The benzimidazole \textit{66} was prepared in two steps by coupling \textit{65} with 4-nitro-1H-pyrazole-3-carboxylic acid under amidation conditions, followed by heating in AcOH. The nitro group of \textit{66} was reduced to the amine via hydrogenation under palladium catalyzed conditions to give \textit{67}, a parallel-enabled scaffold. The desired compounds \textit{68}, \textit{69} and \textit{70} were prepared from \textit{67} using standard amide and urea coupling methods.

The design and synthesis of analogs \textit{69} and \textit{70} was performed to explore a possible SAR cross-over between three potent Aurora kinase scaffolds: danusertib, hesperadin and AT-9283. We envisioned the feasibility of using the headgroups of hesperadin and danusertib for AT-9283 analogs since these compounds all target the same ATP binding pocket with roughly the same defined molecular binding regions as shown in Figure 2.11. Ideally, if compounds maintain potency we could repurpose either the danusertib or hesperadin SAR that has already been performed in our lab when designing new AT-9283 analogs.

AT-9283 was found to be a highly potent inhibitor of \textit{T. brucei brucei} with an EC\textsubscript{50} of 40 nM (tested in the old CTB assay), and therefore it was prepared in quantities sufficient for mouse infection studies. The scale-up synthesis of AT-9283 was also accomplished using the route shown in Scheme 2.5.\textsuperscript{99}
Figure 2.11. Cross-over strategy between three Aurora kinase inhibitors. Molecular regions are defined as follows: a) solvent exposed region, b) kinase hinge region and c) the headgroup region.
2.6.2 Biological testing of AT-9283 in mice

Importantly an estimated 14-fold decrease in parasitemia after 5 days post-infection was observed, and the treated mice lived 36 hours longer than the control mice (experimental details are provided in Appendix 3). This data supports the concept that Aurora kinase inhibitors, in particular AT-9283, are viable tool compounds to reduce parasitemia in mice.

2.6.3 Discussion for AT-9283 scaffold

The human Aurora kinase inhibitor AT-9283 was shown to be a very potent inhibitor against *T. b. brucei* cell cultures. In addition, this Aurora kinase inhibitor prolonged the life of infected mice by 36 hours and reduced by 13.6 fold the parasitemia loading by day 5 post-infection. This is indeed encouraging and provided some proof of concept that aurora kinase inhibitors can indeed reduce parasitemia in mice. The screening of the cross-over analogs is still pending at this time, and the data will thus be reported later. Further analog design work using this scaffold is warranted in order to improve the selectivity over human Aurora and host cell toxicity.
2.7 Summary

To sum up, this chapter has shown that repurposing human Aurora kinase inhibitors, in particular danusertib and AT-9283, for the development of anti-trypanosomal drugs is a feasible approach. We observed cellular growth inhibition with both chemotypes, though a detailed SAR was developed only for the danusertib scaffold to date. We have observed some cellular selectivity for the parasite cells over mammalian cells. Additional efforts have been described in providing tool compounds to confirm molecular mechanism of action for danusertib, as well as a promising in vivo study of the AT-9283 as an additional Aurora kinase inhibitor lead scaffold.
Chapter 3: Design, synthesis and evaluation of TbrPDEB1/B2 inhibitors
3.1 Design, synthesis and evaluation of tadalafil analogs against TbrPDEB

3.1.1 Rationale for synthesis of tadalafil analogs

It has been shown both by our lab\textsuperscript{85} and by others\textsuperscript{100} that there are some human PDE inhibitors and their respective analogs show a diverse degree of inhibition towards the parasitic enzymes. The first synthetic efforts on this project were on tadalafil, a human PDE5 inhibitor that was inactive when tested against TbrPDEB\textsubscript{1} at 100 µM drug concentrations. However, this data did not discourage us, mostly due to the extensive medicinal chemistry knowledge on human PDE5 inhibitors and also due to the fact the tadalafil scaffold has been investigated by other groups as antimalarial lead compounds\textsuperscript{101} and as trypanocides.\textsuperscript{102} Therefore, we decided to explore this chemotype and a variety of tadalafil analogs were hence pursued. To aid our synthetic efforts we initially studied the interactions of tadalafil with human PDE5 which have been studied in the past.\textsuperscript{76} Based on the information from the structure of the catalytic domain of PDE5 with bound tadalafil, the benzo[1,3]dioxane moiety is pointing into a hydrophobic pocket, and according to the homology modeling of the \textit{T. brucei} enzymes, this pocket is predicted to be

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{tadalafil_position.png}
\caption{Putative position of tadalafil in the TbrPDEB\textsubscript{1} binding site.}
\end{figure}
This pocket was named the P-(or parasite) pocket and has been identified first by X-ray crystallography to exist in the *Leishmania major* PDE homolog, LmjPDEB1. The binding site has a glutamine residue, which is conserved across all known PDEs, that interacts with the N-H of the indole. In addition, the Western end of tadalafil is directed in a small lipophilic sub-pocket which is flanking the metal binding pocket in the human enzyme. Based on our homology model of the parasitic enzyme there are a number of polar amino acids in this region. Therefore, we designed our analogs by taking into account the interactions that we thought would exist between tadalafil and the TbrPDEB1 binding site as shown in Figure 3.1.

### 3.1.2 Synthesis and evaluation of the tadalafil analogs

Initial synthetic efforts on the tadalafil scaffold explored each of these regions. A number of analogs were prepared via the route shown in Scheme 3.1. The treatment of the appropriate tryptophan methyl ester with the desired aldehydes under Pictet-Spengler conditions afforded intermediates 71. The next step was the acylation of 71 with chloroacetyl chloride using triethylamine as a base to give the desired chloroacetyl derivatives 72 in good yield. The piperazinedione analogs 73 were obtained by the reaction of 72 with methyl amine in refluxing methanol. The N-methylated compounds 74a and 74b were obtained by the methylation of tadalafil and its C6 epimer via sodium hydride and methyl iodide. The same synthetic steps shown in Scheme 3.1 were used to deliver the brominated analog 75, starting from the commercially available 5-bromotryptophan.
Scheme 3.1 Conditions: (a) R₂-CHO, CH₂Cl₂, CF₃COOH, 0 °C to rt; (b) chloroacetyl chloride, Et₃N, CH₂Cl₂; (c) CH₃NH₂-HCl, MeOH, Et₃N, reflux; (d) NaH, MeI, THF. (Refer to Table 3.1 for R₂)

Scheme 3.2 Conditions: (a) 1H-pyrazol-5-ylboronic acid, aq. Na₂CO₃, Pd(dppf)Cl₂, dioxane, 90 °C, overnight.
The reaction of 75 under Suzuki conditions provided the pyrazole analog 84 shown in Scheme 3.2. The selection of this particular pyrazole moiety from the plethora of available boronic esters or acids was done based on our understanding of the targeted region in the enzyme, namely a small polar pocket that was adjacent to the metal binding site. The pyrazole side chain seemed ideal due to its small size and H-bond donor acceptor functionality that was expected to interact with the polar pocket.

We also wished to explore the solvent-exposed region and this was done via the truncation of the diketopiperazine moiety. Treatment of intermediates 76a-79a with ammonium hydroxide provided the desired tricyclic analogs 76-79 (Scheme 3.3).

Other analogs synthesized were designed to explore the putative P-pocket region and this was done via the reaction sequence shown in Scheme 3.4. To obtain an intermediate with a pendant primary amine (80), CBZ-glycinal was utilized in the Pictet-Spengler reaction, followed by N-acylation and cyclization to give 73a which was obtained from the sequence shown in Scheme 3.1. The CBZ protecting group was removed via catalytic hydrogenation to give the library-enabled amine template 80 which could be either acylated to give the final products 81 or 82 or alkylated to provide 83 (Scheme 3.4).
Scheme 3.4 Conditions: (a) H₂, Pd/C, EtOH, 3h; (b) Ac₂O, DIEA, THF, 24h, rt; (c) 3-methyl-1H-pyrazole-4-carboxylic acid, EDC, Et₃N, HOBt, CH₂Cl₂; (d) nicotinaldehyde, NaBH(OAc)₃, THF/DMF, 48 h.

Scheme 3.5 Conditions: (a) piperonal, CH₂Cl₂, CF₃COOH, 0 ºC to rt; (b) acetyl chloride, Et₃N, THF, rt.
These analogs were designed to probe the steric accessibility of the P-pocket, and also to facilitate some possible interactions with the putative H-bond donors/acceptors present.

A final analog, 85, was prepared by reacting tryptamine with piperonal under Pictet-Spengler conditions followed by N-acylation (Scheme 3.5). This analog was designed to test the requirements for the presence of the diketopiperazine ring.

The tadalafil analogs were screened against TbrPDEB1 and the data are summarized in Tables 3.1 and 3.2. From Table 3.1 we can see that the tricyclic analogs showed only weak activity. In addition, 87 the diastereomer of tadalafil displayed almost 40% inhibition at 100 µM against TbrPDEB1 whereas tadalafil showed no activity at the same concentration. Compound 85 displayed very low activity (17% inhibition). Most of the compounds shown in Table 3.2, including those analogs that probed the P-pocket, show low-to-modest potency. Compound 84 was a notable exception, as it was designed to explore the small polar pocket adjacent to the metal binding region. Compound 84 showed 72% inhibition at 10 µM against TbrPDEB1, yet an IC₅₀ for this particular analog was not obtained since it displayed poor solubility at the higher concentrations required for a reliable dose-response experiment.

3.1.3 Summary: tadalafil-based inhibitors of TbrPDEB1

In summary, the work on the tadalafil scaffold was initiated to provide a broad overview of its structure-activity relationships. The vast majority of analogs showed a weak inhibition profile, with one notable exception, compound 84. This knowledge acquired from the fairly flat SAR and lack of potency informed us that this chemotype does not hold a strong promise as
initially hoped for TbrPDEB1 inhibitors. Our next focus was then shifted onto the more promising PDE4 inhibitor chemotypes.\textsuperscript{85}

**Table 3.1** Potencies of tricyclic tadalafil analogs against TbrPDEB1.

<table>
<thead>
<tr>
<th>Compd</th>
<th>$R^2$ config</th>
<th>$R^2$</th>
<th>amide config</th>
<th>TbrPDEB1 (% inh)\textsuperscript{a}</th>
</tr>
</thead>
<tbody>
<tr>
<td>76</td>
<td>$\alpha$</td>
<td>$\alpha$</td>
<td>$\alpha$</td>
<td>19.5±14.5</td>
</tr>
<tr>
<td>77</td>
<td>$\beta$</td>
<td>$\alpha$</td>
<td>$\alpha$</td>
<td>0</td>
</tr>
<tr>
<td>78</td>
<td>$\alpha, \beta$</td>
<td>CH$_3$</td>
<td>$\alpha,\beta$</td>
<td>13.3±2.2</td>
</tr>
<tr>
<td>79</td>
<td>$\alpha,\beta$</td>
<td>$\alpha$</td>
<td>$\alpha$</td>
<td>22.6±7.3\textsuperscript{b}</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Inhibitor concentration 100.0 µM. \textsuperscript{b}Inhibitor concentration 10.0 µM.
Table 3.2 Tadalafil analogs tested against TbrPDEB1.

<table>
<thead>
<tr>
<th>Compound</th>
<th>R²</th>
<th>R³</th>
<th>R¹</th>
<th>TbrPDEB1 (%inh)(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tadalafil(86)</td>
<td></td>
<td></td>
<td>H</td>
<td>0(^b)</td>
</tr>
<tr>
<td>74a</td>
<td>CH₃</td>
<td>H</td>
<td></td>
<td>6.9±6.9</td>
</tr>
<tr>
<td>75a</td>
<td>H</td>
<td>Br</td>
<td></td>
<td>11.8±5.8</td>
</tr>
<tr>
<td>75</td>
<td>H</td>
<td>Br</td>
<td></td>
<td>11.57±6</td>
</tr>
<tr>
<td>84</td>
<td>H</td>
<td></td>
<td>1H-pyrazol-3-yl</td>
<td>71.8±5.8</td>
</tr>
<tr>
<td>74b</td>
<td>CH₃</td>
<td>H</td>
<td></td>
<td>19.7±2.1</td>
</tr>
<tr>
<td>87</td>
<td>H</td>
<td>H</td>
<td></td>
<td>38.8±3.2(^b)</td>
</tr>
<tr>
<td>73a</td>
<td>NHCbz</td>
<td>H</td>
<td>H</td>
<td>21.2±1.3</td>
</tr>
<tr>
<td>73b</td>
<td></td>
<td>H</td>
<td>H</td>
<td>9.4±9.4(^b)</td>
</tr>
<tr>
<td>81</td>
<td>NHAc</td>
<td>H</td>
<td>H</td>
<td>0</td>
</tr>
<tr>
<td>82</td>
<td></td>
<td>H</td>
<td>H</td>
<td>0</td>
</tr>
<tr>
<td>83</td>
<td></td>
<td>H</td>
<td>H</td>
<td>7.89±2.9</td>
</tr>
</tbody>
</table>

\(^a\)Inhibitor concentration 10.0 μM. \(^b\)Inhibitor concentration 100.0 μM.
3.2 Exploring the hPDE4 inhibitors for activity against TbrPDEB1/B2

The lack of success with repurposing hPDE5 inhibitors led us to refocus our synthetic efforts on the more promising hPDE4 inhibitors. This new strategy was further supported by our knowledge that TbrPDEB1 and B2 mainly regulate intracellular cyclic adenosine monophosphate (cAMP) levels. Given the knowledge that the hPDE4 family hydrolyzes only cAMP and also that hPDE4 inhibitors from our initial benchmarking paper showed inhibition of the TbrPDEB1 and B2 at various levels, we prioritized this class of inhibitors. The initial screening of a variety of piclamilast analogs had already shown some modest inhibition of TbrPDEB1 and B2 and allowed us to develop a preliminary SAR on this hPDE4 inhibitor. Our synthetic efforts were further aided by Dr. Zhouxi Wang who developed a homology model of TbrPDEB1 (Figure 3.2) to allow an understanding of the compound-enzyme interactions. Thus, we were hoping that differences observed via this model and complemented by SAR between the parasitic and the human enzymes would ultimately lead to the design of potent and selective TbrPDEB inhibitors.

To date, we have focused on two classes of hPDE4 inhibitors: piclamilast and GSK-256066. A driving hypothesis for analog design is, as previously mentioned, the presence of the "P-pocket" and this region is illustrated in our homology model of TbrPDEB1 (Figure 3.2). Since this feature is not present in human PDEs, it could represent a means to achieve selectivity for the parasite enzymes over the human homologs. Our working model consists of the P-pocket plus other regions of importance to PDE inhibitor binding: the metal binding site (termed the "headgroup" region), a pocket containing the conserved purine-binding glutamine residue and the hydrophobic clamp (Q pocket).
Figure 3.2 (A) The crystal structure of human PDE4B complexes with piclamilast (PDB ID: 1XM4) compared to (B) The predicted pose for piclamilast in the comparative model of TbrPDEB1; (C) The TbrPDEB1 homology model (pink) superimposed with hPDE4 (gray). Non-conserved binding site residues are shown as sticks (residues of TbrPDEB1 colored magenta). (D) View of the P-pocket in the TbrPDEB1 homology model structure, viewed from the opposite face. Reprinted with permission from Bland, N. D.; Wang, C.; Tallman, C.; Gustafson, A. E.; Wang, Z.; Ashton, T. D.; Ochiana, S. O.; McAllister, G.; Cotter, K.; Fang, A. P.; Gechijian, L.; Garceau, N.; Gangurde, R.; Ortenberg, R.; Ondrechen, M. J.; Campbell, R. K.; Pollastri, M. P., Pharmacological validation of Trypanosoma brucei phosphodiesterases B1 and B2 as druggable targets for African sleeping sickness. J Med Chem. 2011, 54 (23), 8188-94. Copyright (2011) American Chemical Society.
3.3 Piclamilast as a new starting point for analog design for TbrPDEB1/B2

3.3.1 Piclamilast and a special focus on the "P-pocket"

The molecular regions for the piclamilast chemotype are defined in Figure 3.3.

Some of these molecular regions have been extensively studied in our lab, and the preliminary SAR of this chemotype for both the headgroup and core regions have been established.

A group of European researchers revealed a very potent nanomolar inhibitor of TbrPDEB1 which putatively fills the P-pocket (Figure 3.4). Thus, we sought to build upon the
established P-pocket SAR by pursuing a set of analogs to further explore this enticing region to develop more potent analogs.

![Compound A](image)

**Compound A**

TbrPDEB1 IC$_{50}$: 3.98 nM

**Figure 3.4** Tetrahydrophthalazinone (Compound A), a very potent TbrPDEB1 inhibitor.

The first synthetic efforts on this chemotype were aimed at designing piclamilast analogs that would explore the P-pocket by examining the extension of the chain and polarity, and thus further filling the SAR gaps on this region. To achieve our desired goals we had to first optimize the chemistry and synthesize the library enabled scaffold shown in **Scheme 3.6**. The first step was the reaction of methyl 3-hydroxy-4-methoxybenzoate under Mitsunobu conditions to give intermediate 91. Then the ester of 91 was hydrolyzed to the acid product 92. The acid was subsequently converted to the acid chloride followed by amidation with 4-amino-3,5-dichloropyridine and deprotection of the phthalimide to give the free amine intermediate 93. This amine intermediate represents a parallel-enabled scaffold for library synthesis.

The reaction of 93 with various electrophiles (e.g. acid chlorides, sulfonyl chlorides, isocyanates) provided the desired analogs shown in **Figure 3.5**. A couple of diverse analogs (having polar/non-polar groups) were made to assess the polarity of this pocket, and only a modest improvement in potency was observed for 100 (**Figure 3.5**). The more lipophilic
Scheme 3.6 General route for piclamilast analogs for the P-pocket. Reagents and conditions: a) 2-(2-hydroxyethyl)isoindoline-1,3-dione, PPh₃, DEAD, toluene, rt, 12h (47%). b) NaOH, H₂O, 90°C, 1h (95%). c) SOCl₂, toluene, reflux, 4h (100%). d) NaH, DMF, 0-10 °C, 1h then 3,5-dichloropyridin-4-amine, 50 °C, 1h. e) H₂NNH₂, MeOH, rt, 12h (44%). f) Reaction of 93 with various electrophiles (see Appendix 2 for specific reagents).

Figure 3.5 P-pocket analogs synthesized via the synthetic route shown in Scheme 3.6. The
compounds that also contained an aromatic moiety (97, 98, 100) were shown to be more active. The data available for these P-pocket analogs combined with the knowledge from Compound A (Figure 3.4) suggest that further explorations should be perhaps aimed at increasing the size of the carbon linker from 2 (e.g. our P-pocket analogs) to 4 (Compound A) to achieve a further increase in activity. Another idea besides varying the size of the linker is to also look at further substitutions on the aromatic ring aimed at better filling the P-pocket.

3.3.2 Summary: piclamilast-based inhibitors of TbrPDEB1

The extensive SAR that was performed on the piclamilast chemotype via synthesis of a large diverse set of analogs, either by myself or other members of our group has not yet revealed better analogs than piclamilast. The P-pocket compounds described in this work (94-100) were designed to fill in this pocket but did not provide any notable gain in potency. Most of the analogs synthesized showed a decrease in potency (e.g. 94, 95, 96, 97) when comparing with the original hit compound piclamilast. This could suggest that these analogs might be binding in a different way.
3.4 GSK256066 scaffold exploration

3.4.1 Selection of GSK256066 for SAR determination

This compound is an exceedingly potent human PDE4 inhibitor (8 pM)\textsuperscript{107} that showed weak activity against TbrPDEB1 (Figure 3.6, IC\textsubscript{50}=24.6 µM). However, based on our experience with the really tight SAR for the PDE4 compounds (e.g. comparing roflumilast, which is inactive- Table 1.3, and piclamilast, which is 4.6 µM,) it seemed reasonable that the 24.6 µM GSK256066 could yield some potent compounds with some SAR development work.

![Diagram of GSK256066](image)

**Figure 3.6** Defining molecular regions. The parasite-unique “P-pocket” is shown, in comparison to the smaller hydrophobic pocket in hPDE4.

We guided our GSK256066 chemotype exploration using our understanding of the protein-compound interactions highlighted in Figure 3.6. Our working model, similar to that of
piclamilast, consists of the P-pocket, plus other regions of established importance to PDE inhibitor binding: the metal binding site (termed the “headgroup” region), a solvent-exposed region (not present in piclamilast), a pocket containing the conserved purine-binding glutamine residue and the hydrophobic clamp. Armed with this information, we explored the SAR for this quinoline-3-carboxyamide scaffold.

3.4.2 Synthesis of GSK256066 analogs

The first step was to scale up the desired intermediates (105, 106) with the general structure shown in Scheme 3.7. Then, with gram quantities in hand we could focus on preparing either singleton analogs or small libraries. The desired analogs were synthesized as shown in Scheme 3.7. The R₁ substituent was introduced at the beginning of the synthesis with the appropriate aniline 102 which was condensed with diethyl ethoxymethylene malonate to give 103. The cyclization of 103 in diphenyl ether gave the ester 104. The ester was hydrolyzed to the acid using sodium hydroxide. Then the resulting acid product was dichlorinated using thionyl chloride at both the acid moiety and at the 4-position. Quenching with aqueous ammonia afforded the primary carboxyamide 105. The next step was to introduce the desired functionality at R₂ which was achieved by refluxing 105 with the desired amine to give 106. The initial analogs were focused on exploring the solvent-exposed region shown in Figure 3.6. These analogs were synthesized via the reaction of a large variety of boronic acids, aryl thiols and amines with the iodo-substituted template 106 using Suzuki couplings to give biphenyl analogs 108, other palladium-catalyzed methodologies for aryl thiols 107 and Buchwald-Hartwig
chemistry to access substituted amine analogs 109. Additional analogs were synthesized via Scheme 3.8.

**Scheme 3.7**

a) EtOCH=C(CO₂Et)₂, 100°C, 1h (80-98%).
b) Ph₂O, 250 °C, 1h (81-100%).
c) NaOH, EtOH, reflux, 1h (80-90%).
d) SOCl₂, DMF, 80 °C, 2h (100%)
e) NH₄OH, rt, 12h (61-92%).
f) NH₂-R₂, MeCN, 80 °C, 24h or NH₂-R₂, NaH, DMF, 50 °C, 2h (61-80%).
g) R₃-SH, Pd₂(dba)₃, DPEphos, KOtBu, toluene, 170 °C, M.W., 30 minutes (70-85%).
h) Oxone, DMF, rt, 12-24h (70-90%).
i) R₄-B(OH)₂, Na₂CO₃, Pd(dppe)Cl₂, dioxane, 130 or145 °C, M.W., 20 minutes (20-70%).
j) R₅-NH₂, Pd(dppe)Cl₂, (oxybis(2,1-phenylene))bis(diphenylphosphine), KOtBu, dioxane, 150 °C, M.W., 20 minutes (10-15%).
Reaction of 110 with (3-(methoxycarbonyl)phenyl)boronic acid using Suzuki coupling conditions provided the desired product 114 and the byproducts 111 and the dehalogenated 112. Then, the final step required heating of 113 or 114 with the desired amine or alcohol to give the final products 115 or 116 (Scheme 3.8).

**Scheme 3.8**

a) (3-(methoxycarbonyl)phenyl)boronic acid, Na₂CO₃, Pd(dppf)Cl₂, dioxane, 150 °C, M.W., 10 minutes. b) (3-(oxazol-2-yl)phenyl)boronic acid, Na₂CO₃, Pd(dppf)Cl₂, dioxane, 120 °C, M.W., 20 minutes (41%). c) CH₃CN, H₂N-R 80°C, 24-48h or CH₃CN, HO-R, DBU, 150°C, 40 min. d) CH₃CN, H₂N-R, 80°C, 24-48h.
3.4.3 Analogs & SAR interpretation for the "solvent-exposed region"

We first began our SAR studies for the GSK256066 chemotype by testing analogs that have been previously explored for the human PDE4 enzyme. Thus, we would initially repurpose published SAR to gain a better understanding of the differences between the parasitic enzymes and human ones. In addition, we were hoping to be able to also reduce the molecular weight of the compounds and thus increase ligand efficiency by determining the optimal fragment size needed for activity in this region. The region targeted for synthetic modifications is highlighted in Table 3.3. Thus, the first synthetic efforts were focused on small systematic changes to the solvent-exposed region of the GSK256066 scaffold (Figure 3.6) by exploring the effect of various functional groups such as amide, ester, acid, unsubstituted phenyl, or by complete removal of the phenyl group. The data shown in Table 3.3 indicates that a substituted phenyl is needed for TbrPDE potency as the unsubstituted 127 analog is essentially inactive. Noteworthy is the fact that from the published SAR against human PDE4 we knew that this region is not vital for activity against the human PDE4 enzyme; nonetheless, the SAR for TbrPDEB1 has revealed that this region is indeed important for activity against the parasite. In the parasite enzyme, this region of the molecule seems to favor aromatic rings that are substituted in the meta position with hydrogen bond donors and acceptors (e.g. 122, 123, 125).

Our next goal was to evaluate the linker between the core and the solvent-exposed region. Since the ester analog 122 was among the most potent ones we decided to retain the ester functionality and explore linkers other than sulfonyl. Therefore, we pursued the synthesis of three other linkers: sulfide (126), biaryl (132) and amine (131) (Table 3.3). The sulfide linker resulted in complete loss of activity, the amine linker showed moderate activity whereas the
biaryl linker (132) was essentially equipotent with the sulfone (122) linker. This information allowed us to prioritize our next set of analogs.

The fact that the matched biaryl analog of 122 was equipotent opened a new avenue for us, since it was now easier to access a larger set of analogs via standard Suzuki couplings. We could now rapidly explore a complete SAR of this region. The next immediate goal was the synthesis of a large set of analogs that would provide detailed knowledge of the preferred substitution (meta, ortho, para) and the polarity profile of this region.

It was interesting to see from the initial set of analogs that both the ester (122) and the acid (123) display a similar activity profile. This observation made us consider a possible dual or different binding mode of the acid vs. ester, and made us hypothesize that we should see an increase in potency if both functionalities were present on the phenyl. To test this hypothesis we synthesized the analogs 149 (diester), 150 (monoacid and monoester) and 151 (diacid) (Table 3.4). In addition, we also wanted to further explore in the meta position the effect of bioisosteres of both the acid and the ester, and as such analogs like 142 (tetrazole) or 152 (oxazole) were also synthesized.

The set of analogs also included larger heterocycles, such as quinoline (140) or indole (141). We were able to synthesize a large number of analogs exploring a diverse set of substituents, and most of these analogs are detailed in Table 3.3, Table 3.4 and is summarized in the SAR Figure 3.7. Some key SAR findings for TbrPDEB1 enzyme potency in the western end region are:

1. *Meta* substitution on the aromatic ring is needed for potency (*e.g.* ester, tetrazole, other small heterocyles).
2. *Ortho* substitution resulted in a significant decrease in activity (*e.g.* 146, TbrPDEB1 IC$_{50}$ ~30 µM) nor was substitution of the *para* position tolerated at all (*e.g.* 147).

3. The best linkers were SO$_2$ (*e.g.* 122) and biaryl linker (*e.g.* 132) whereas N was tolerated (131) and S (126) resulted in a complete loss of activity.

4. Other active fragments were discovered such as pyrazole (138) indazole (133) and indole (141).

5. Disubstituted phenyl decreases activity (149, 150, 151).

6. Unsubstituted phenyl (127) leads to a dramatic decrease in potency.

---

**Figure 3.7** SAR of the solvent-exposed region.
Table 3.3 Potencies of western end GSK256066 analogs against TbrPDEB1.

<table>
<thead>
<tr>
<th>Compound</th>
<th>R₁</th>
<th>X</th>
<th>TbrPDEB1 (% inh)</th>
<th>TbrPDEB1 (IC₅₀ µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSK256066</td>
<td><img src="image1" alt="Structure" /></td>
<td><img src="image2" alt="Structure" /></td>
<td>52.7±6.7</td>
<td>24.6±3.6</td>
</tr>
<tr>
<td>124</td>
<td><img src="image3" alt="Structure" /></td>
<td><img src="image4" alt="Structure" /></td>
<td>72.3±0.3</td>
<td>26.0±7</td>
</tr>
<tr>
<td>125</td>
<td><img src="image5" alt="Structure" /></td>
<td><img src="image6" alt="Structure" /></td>
<td>70.3±3</td>
<td>14.5±4.2</td>
</tr>
<tr>
<td>122</td>
<td><img src="image7" alt="Structure" /></td>
<td><img src="image8" alt="Structure" /></td>
<td>77±4.7</td>
<td>3.4±0.4</td>
</tr>
<tr>
<td>123</td>
<td><img src="image9" alt="Structure" /></td>
<td><img src="image10" alt="Structure" /></td>
<td>92.6±1.5</td>
<td>2.7±1.1</td>
</tr>
<tr>
<td>127</td>
<td><img src="image11" alt="Structure" /></td>
<td><img src="image12" alt="Structure" /></td>
<td>21.2±15.8</td>
<td>-</td>
</tr>
<tr>
<td>126</td>
<td><img src="image13" alt="Structure" /></td>
<td><img src="image14" alt="Structure" /></td>
<td>2.8⁹</td>
<td>-</td>
</tr>
<tr>
<td>131</td>
<td><img src="image15" alt="Structure" /></td>
<td><img src="image16" alt="Structure" /></td>
<td>79.7±6.2</td>
<td>17.3⁹</td>
</tr>
<tr>
<td>132</td>
<td><img src="image17" alt="Structure" /></td>
<td><img src="image18" alt="Structure" /></td>
<td>96±5.7</td>
<td>5.4±0.6</td>
</tr>
</tbody>
</table>

⁹ number of replicates (n) = 1
Table 3.4 Potencies of western end GSK256066 analogs against TbrPDEB1.

<table>
<thead>
<tr>
<th>Compound</th>
<th>R₁</th>
<th>TbrPDEB1 (% inh)</th>
<th>TbrPDEB1 (IC₅₀ µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>137</td>
<td><img src="image1" alt="Structure" /></td>
<td>34±18.1</td>
<td>25±1.6</td>
</tr>
<tr>
<td>135</td>
<td><img src="image2" alt="Structure" /></td>
<td>74.6±2.1</td>
<td>12±4.6</td>
</tr>
<tr>
<td>133</td>
<td><img src="image3" alt="Structure" /></td>
<td>83.7±9.1</td>
<td>3.8±0.4</td>
</tr>
<tr>
<td>134</td>
<td><img src="image4" alt="Structure" /></td>
<td>42.9±16</td>
<td>32.4ᵃ</td>
</tr>
<tr>
<td>141</td>
<td><img src="image5" alt="Structure" /></td>
<td>94ᵃ</td>
<td>4.9±0.8</td>
</tr>
<tr>
<td>140</td>
<td><img src="image6" alt="Structure" /></td>
<td>49.1ᵃ</td>
<td>-</td>
</tr>
<tr>
<td>142</td>
<td><img src="image7" alt="Structure" /></td>
<td>94ᵃ</td>
<td>3.8±0.9</td>
</tr>
<tr>
<td>155</td>
<td><img src="image8" alt="Structure" /></td>
<td>53.9±15.2</td>
<td>-</td>
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</tbody>
</table>

ᵃ number of replicates (n) = 1
Table 3.4, continued. Potencies of western end GSK analogs against TbrPDEB1.

<table>
<thead>
<tr>
<th>Compound</th>
<th>R&lt;sub&gt;1&lt;/sub&gt;</th>
<th>TbrPDEB1 (% inh)</th>
<th>TbrPDEB1 (IC&lt;sub&gt;50&lt;/sub&gt; µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>136</td>
<td></td>
<td>12.8±5.2</td>
<td>-</td>
</tr>
<tr>
<td>138</td>
<td></td>
<td>84±5</td>
<td>8.9±1.4</td>
</tr>
<tr>
<td>139</td>
<td></td>
<td>19&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-</td>
</tr>
<tr>
<td>143</td>
<td></td>
<td>13±14</td>
<td>-</td>
</tr>
<tr>
<td>145</td>
<td></td>
<td>66.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-</td>
</tr>
<tr>
<td>144</td>
<td></td>
<td>62.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-</td>
</tr>
<tr>
<td>147</td>
<td></td>
<td>4.7±0.9</td>
<td>-</td>
</tr>
<tr>
<td>146</td>
<td></td>
<td>50.3±9.7</td>
<td>-</td>
</tr>
</tbody>
</table>

<sup>a</sup> number of replicates (n) = 1
Table 3.4, continued. Potencies of western end GSK256066 analogs against TbrPDEB1.

<table>
<thead>
<tr>
<th>Compound</th>
<th>R&lt;sub&gt;1&lt;/sub&gt;</th>
<th>TbrPDEB1 (% inh)</th>
<th>TbrPDEB1 (IC&lt;sub&gt;50&lt;/sub&gt; µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>148</td>
<td></td>
<td>27.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-</td>
</tr>
<tr>
<td>150</td>
<td></td>
<td>40±28</td>
<td>-</td>
</tr>
<tr>
<td>151</td>
<td></td>
<td>15.5±2.2</td>
<td>-</td>
</tr>
<tr>
<td>149</td>
<td></td>
<td>55±7.6</td>
<td>-</td>
</tr>
<tr>
<td>152</td>
<td></td>
<td>66.5±39.7</td>
<td>6.3&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>153</td>
<td></td>
<td>32.5±15.3</td>
<td>-</td>
</tr>
<tr>
<td>154</td>
<td></td>
<td>82.5±2.5</td>
<td>4.3±2.5</td>
</tr>
<tr>
<td>156</td>
<td></td>
<td>30.8±12.5</td>
<td>-</td>
</tr>
</tbody>
</table>

<sup>a</sup> number of replicates (n) = 1
The analogs synthesized allowed us to understand better what kind of functionalities are preferred and/or tolerated by the enzyme in the solvent-exposed region. However, one question that arises is why we would notice such dramatic changes in activity by simply changing functional groups or exploring the best position for substitutions on the phenyl, if this region of the molecule only points towards solvent?

The next step was the exploration of the headgroup region, and this region has been previously explored in our lab for the piclamilast chemotype.

3.4.4 Analogs and SAR interpretation for the "headgroup region"

The design of analogs for the headgroup region was performed by keeping the western end constant with the meta-ester functionality or the methyl indazole fragment, which were shown to be equipotent. What is more, we wanted to utilize the established human PDE4 SAR to guide our synthetic efforts, seeking to reduce activity at hPDE4 while improving activity at TbrPDEB1. This approach could give us some leverage to gain both potency and selectivity.

The first step involved the synthesis of analogs that would test for the best position for substitutions on the phenyl ring (Table 3.5). Thus, the methoxy functional group of the original lead was moved from the meta to the ortho (166) and para (173) positions, and then removed completely (169). In addition, since in hPDE4 of this region there is a metal involved in binding via the methoxy oxygen, we replaced the oxygen atom with carbon (174). In the meta position we also explored the effect of shorter and longer substituents, such as a methyl (171) and ethoxy (170). In addition, based on our past experience with this region we attempted to cross-over SAR
knowledge from the piclamilast chemotype. Thus, analog 162 was also synthesized containing the headgroup of piclamilast.

Another hypothesis we wished to test was the importance of the NH headgroup linker. When this is modified a detrimental effect is observed for human PDE4 potency because an intramolecular hydrogen bond between the NH and the carbonyl group of the 3-carboxamide (Figure 3.6 and Figure 3.11) is no longer possible. Thus, the synthesis of the methylated amine 165 was undertaken to first see if the SAR further translates between the human and the parasitic enzymes.

Other analogs involved truncation of the entire headgroup (112), further extension towards the metal atoms in the pocket by changing the phenyl to a benzyl (133 to 164), replacement of the aromatic functionality with an aliphatic ring (179), plus other miscellaneous analogs (Table 3.6). Some key SAR findings for the headgroup region in the TbrPDEB1 enzyme from Table 3.5 and Table 3.6 are summarized below and highlighted in the SAR Figure 3.8:

1. Similar to the hPDE4 binding site the meta substitution on the aromatic ring was required for potency (e.g. methyl, ethyl, methoxy, ethoxy).

2. A methyl ether moiety is required in the meta position for increased hPDE4 potency 107; this is apparently not required for the parasitic enzyme (171, 174) activity. Similar potency against TbrPDEB1 is observed for both m-methoxy (133) and m-ethyl (174). This is a possible differentiating factor in SAR that can potentially be leveraged for selectivity against TbrPDE over hPDE4.

3. Substitution in the ortho position was tolerated (166), whereas the para position resulted in a dramatic loss in activity (173).
4. The best linker was NH and other linkers (e.g. C-C or O) resulted in almost a complete loss of activity.

5. Another weakly active fragment was discovered, the cyclopentyl amine (179).

6. Replacement of *meta* substituted phenyl with benzyl decreases activity (163, 164).

7. Unsubstituted phenyl (169) leads to a dramatic decrease in potency.

*Figure 3.8* SAR of the headgroup region.
Table 3.5 Potencies of headgroup GSK256066 analogs against TbrPDEB1.

![Chemical structure of a headgroup analog](image)

<table>
<thead>
<tr>
<th>Compound</th>
<th>R&lt;sub&gt;2&lt;/sub&gt;</th>
<th>TbrPDEB1 (%) inh</th>
<th>TbrPDEB1 (IC&lt;sub&gt;50&lt;/sub&gt; µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>169</td>
<td></td>
<td>27.3±1.1</td>
<td>-</td>
</tr>
<tr>
<td>170</td>
<td></td>
<td>82.1±23.5</td>
<td>3.9&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>171</td>
<td></td>
<td>84.6±22.1</td>
<td>3.5±1.1</td>
</tr>
<tr>
<td>172</td>
<td></td>
<td>85.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.4±0.1</td>
</tr>
<tr>
<td>173</td>
<td></td>
<td>34.4±5.2</td>
<td>-</td>
</tr>
<tr>
<td>174</td>
<td></td>
<td>67.4±13.1</td>
<td>3.1±0.6</td>
</tr>
<tr>
<td>162</td>
<td>Cl</td>
<td>40.7±4</td>
<td>8.2&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>166</td>
<td></td>
<td>52.7±31.2</td>
<td>7.5&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> number of replicates (n) = 1
Table 3.6 Potencies of headgroup GSK analogs against TbrPDEB1.

<table>
<thead>
<tr>
<th>Compound</th>
<th>( R_2 )</th>
<th>TbrPDEB1 (% inh)</th>
<th>TbrPDEB1 (IC\textsubscript{50} µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>165</td>
<td></td>
<td>58.5±6.3</td>
<td>-</td>
</tr>
<tr>
<td>111</td>
<td></td>
<td>41.2±10.1</td>
<td>-</td>
</tr>
<tr>
<td>112</td>
<td>( -H )</td>
<td>0.0±0.0</td>
<td>-</td>
</tr>
<tr>
<td>161</td>
<td></td>
<td>81±6.7</td>
<td>25.5( ^a )</td>
</tr>
<tr>
<td>167</td>
<td></td>
<td>30.6±30.2</td>
<td>-</td>
</tr>
<tr>
<td>163</td>
<td></td>
<td>18.5±9.4</td>
<td>-</td>
</tr>
<tr>
<td>164</td>
<td></td>
<td>19.4±14.6</td>
<td>-</td>
</tr>
<tr>
<td>179</td>
<td></td>
<td>80.2±8.4</td>
<td>-</td>
</tr>
</tbody>
</table>

\( ^a \) number of replicates (n) = 1

While most changes followed the human PDE4 SAR, there were two notable exceptions. First, we were surprised that the meta substituents methoxy, ethoxy, methyl and ethyl were
equally active in the parasitic enzyme suggesting that these compounds might not necessarily bind with the metal as anticipated. The second observation was that the cyclopentyl amine analog 179 was active. The latter observation was pursued further and our work is described in the next section in detail.

3.4.4.1 Are we looking at another binding mode?

The idea of another binding mode seemed to gain ground when we noticed that the cyclopentyl analog (179) was active. In Figure 3.9 there is a depiction of the original proposed binding mode and the new possible binding mode supported by a direct comparison with piclamilast. Our new hypothesis suggested that the cyclopentyl amine could now be pointing in

![Figure 3.9 Exploring potential flipped binding mode.](image)
the P-pocket and that the \textit{meta}-methyl ester was directed towards the headgroup region. This seemed plausible for two main reasons. The first was the observation that similar potency against TbrPDEB1 is observed for both \textit{m}-methoxy and \textit{m}-ethyl (132 vs. 174) which seems to disregard that metal binding is involved.

![Chemical Structures](image)

\textbf{Figure 3.10} Compounds synthesized to validate a new binding mode.

The second reason was the drastic changes observed in activity, from high activity (132) to almost no activity (127) by simply removing the \textit{meta} ester substituent, for modifications made for a region we had originally believed to be solvent-exposed. More importantly, this other
binding mode is potentially plausible, since the inhibitor could potentially maintain the critical interactions with the glutamine residue. To validate or invalidate our hypothesis three more analogs were designed and synthesized using a direct comparison with piclamilast.

The regions targeted for modifications are shown in Figure 3.10 and the analogs were synthesized as shown in Scheme 3.8. Since the oxazole \textit{152}, the bioisostere of the ester analog \textit{132}, showed similar potency against TbrPDEB1, we decided to also synthesize two analogs having the oxazole functional group on the western end. We considered this moiety more desirable than the hydrolytically labile ester functional group. The amine linker was replaced with the oxygen since our hypothesis presumed that this replacement will result in at least equal potency. The comparison with the binding mode of piclamilast enforced our idea and also the hope for a possible SAR cross-over between the quinoline and catechol scaffolds (Figure 3.9).

However, after the synthesis and testing of these analogs our hypothesis of a new binding mode was not supported since both \textit{180} and \textit{182} were very weakly active. On the other hand, what we did learn was that the substituted phenyl can be replaced with the cyclopentyl amine which allows for equal potency but a significant decrease in both molecular weight and lipophilicity. Thus, compound \textit{181} (MW: 412.48 and log P: 4.59, vs. \textit{152} MW: 450.48 and log P: 5.69) is compliant with Lipinski's Rule of five which is a useful filter for selecting compounds that are more likely to become orally active drugs in humans. The potency still needs to be improved for this compound and the selectivity versus the human PDE4 enzyme is still pending.
3.4.5 Analogs & SAR interpretation for the "P-pocket" & "Q-pocket"

The next exploratory steps were focused on the P-pocket and Q-pocket (Figure 3.6). The initial design of analogs for these regions, as per our overall repurposing strategy, was first guided by GSK256066 SAR for hPDE4. The P-pocket and Q-pocket compounds were synthesized using the route shown in Scheme 3.7 and are shown in Table 3.7. In the hPDE4 enzyme for the GSK quinoline chemotype the removal of the methyl was detrimental and the increase of the alkyl chain length did not provide an increase in potency. The TbrPDEB1 SAR correlated well with the observed human PDE4B SAR since removal of the methyl decreased activity and increasing of the alkyl chain maintained potency (133, 160).

We then turned our attention to the Q-pocket (Figure 3.6 and Figure 3.11), by specifically methylating the 3-primary carboxamide, a structural change that was shown in the human enzyme to be detrimental for activity. From the human PDE4B crystal structure with the bound GSK quinoline inhibitor we know that the carboxamide sits in a small binding pocket; here one of the NH groups hydrogen bonds to Asn395 and the other NH group binds to the Glu443 via a water molecule (Figure 3.11), and removal of these hydrogen bonding interactions results in a significant decrease in activity. We were interested to see if this was also the case for the TbrPDEB1 enzyme, and so the mono-methylated amide analog 175 and di-methylated analog 176 were prepared (Table 3.7).

The biological data supported the general understanding that removal of the hydrogen bond donors leads to a significant decrease in activity (175, 176). The loss of potency was in the order NH₂ > NH-CH₃ > N-(CH₃)₂. In general the TbrPDEB1 SAR for both the P and Q pockets correlated well with the published hPDE4 SAR for the quinoline chemotype.
Table 3.7 Potencies of P-pocket and Q-pocket GSK256066 analogs against TbrPDEB1.

<table>
<thead>
<tr>
<th>Compound</th>
<th>R₃</th>
<th>R₄</th>
<th>TbrPDEB1 (% inh)</th>
<th>TbrPDEB1 (IC₅₀ µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>158</td>
<td>H</td>
<td>NH₂</td>
<td>84±4.1</td>
<td>14.6ᵃ</td>
</tr>
<tr>
<td>133</td>
<td>CH₃</td>
<td>NH₂</td>
<td>83.7±9.1</td>
<td>3.8±0.4</td>
</tr>
<tr>
<td>160</td>
<td>CH₂CH₃</td>
<td>NH₂</td>
<td>84.2±16.7</td>
<td>4.2ᵃ</td>
</tr>
<tr>
<td>175</td>
<td>CH₃</td>
<td>NHCH₃</td>
<td>33.9±18.8</td>
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</tr>
<tr>
<td>176</td>
<td>CH₃</td>
<td>CH₃</td>
<td>18.2±4.2</td>
<td>-</td>
</tr>
</tbody>
</table>

ᵃ number of replicates (n) = 1
3.4.6 Biochemical vs. cell assay data for most potent GSK analogs

A number of potent GSK256066 analogs identified in the biochemical assay were then tested into cellular assays of trypanocidal activity. The screening data are shown in Table 3.8, 3.9 and 3.10. All the inhibitors were cytotoxic in a dose dependent manner, resulting in micromolar EC\textsubscript{50} values, most of which were comparable to the IC\textsubscript{50} values determined previously against the recombinant TbrPDEB1. The only notable exceptions were compounds 159 (EC\textsubscript{50} of 630 nM) and 170 (EC\textsubscript{50} of 800 nM). Overall, the cell assay data is indicative that cell permeability was maintained with the GSK256066 analogs. In addition, the slight variation that is observed between the biochemical assay and cell assay could be attributed to factors, such as, cell permeability/ compounds accumulation, metabolism in the cell, resistance to breakdown and off target effects. This last possibility is further supported by a hit identified in a high-throughput screen (HTS) performed in collaboration with GlaxoSmithKline (GSK) at GSK-Tres Cantos. The compound, shown in Figure 3.12, has a scaffold similar to the GSK256066 PDE4 inhibitors described in this text and it has an EC\textsubscript{50} of about 100 nM against \textit{T. brucei brucei} cells. Still the mechanism of action is not known.

![Figure 3.12](image-url)
Table 3.8 Summary of dose-response of select GSK256066 analogs showing TbrPDEB1 IC\textsubscript{50} ≤ 12\,\mu M.

![Chemical structure](image)

<table>
<thead>
<tr>
<th>Compound</th>
<th>R\textsubscript{1}</th>
<th>TbrPDEB1 (IC\textsubscript{50}, \mu M)</th>
<th>Cell culture\textsuperscript{1} (EC\textsubscript{50}, \mu M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>122</td>
<td><img src="image" alt="Structure" /></td>
<td>3.4±0.4</td>
<td>7.2±2.4</td>
</tr>
<tr>
<td>132</td>
<td><img src="image" alt="Structure" /></td>
<td>5.8±0.6</td>
<td>2.3\textsuperscript{a}</td>
</tr>
<tr>
<td>133</td>
<td><img src="image" alt="Structure" /></td>
<td>3.8±0.4</td>
<td>5.2\textsuperscript{a}</td>
</tr>
<tr>
<td>135</td>
<td><img src="image" alt="Structure" /></td>
<td>12±4.6</td>
<td>7.8±1.6</td>
</tr>
<tr>
<td>141</td>
<td><img src="image" alt="Structure" /></td>
<td>4.9±0.8</td>
<td>3.8±0.9</td>
</tr>
<tr>
<td>142</td>
<td><img src="image" alt="Structure" /></td>
<td>3.8±0.1</td>
<td>4.5\textsuperscript{a}</td>
</tr>
<tr>
<td>154</td>
<td><img src="image" alt="Structure" /></td>
<td>4.3±0.3</td>
<td>2.7±0.2</td>
</tr>
</tbody>
</table>

\textsuperscript{1}Trypanosoma brucei brucei strain 427. \textsuperscript{a} number of replicates (n) = 1
Table 3.9 Summary of dose-response of select GSK256066 analogs showing TbrPDEB1 IC$_{50}$ < 9µM.

![Chemical structure](image)

<table>
<thead>
<tr>
<th>Compound</th>
<th>$R_1$</th>
<th>$R_2$</th>
<th>TbrPDEB1 (IC$_{50}$ µM)</th>
<th>Cell culture$^1$ (EC$_{50}$, µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>160</td>
<td><img src="image" alt="Structure" /></td>
<td>$\text{CH}_2\text{CH}_3$</td>
<td>4.2$^a$</td>
<td>7.9±3.8</td>
</tr>
<tr>
<td>170</td>
<td><img src="image" alt="Structure" /></td>
<td>$\text{CH}_3$</td>
<td>3.9$^a$</td>
<td>0.8$^a$</td>
</tr>
<tr>
<td>171</td>
<td><img src="image" alt="Structure" /></td>
<td>$\text{CH}_3$</td>
<td>3.5±1.1</td>
<td>5.6±3</td>
</tr>
<tr>
<td>172</td>
<td><img src="image" alt="Structure" /></td>
<td>$\text{CH}_3$</td>
<td>3.4±0.1</td>
<td>2.7±0.2</td>
</tr>
<tr>
<td>174</td>
<td><img src="image" alt="Structure" /></td>
<td>$\text{CH}_3$</td>
<td>3.1±0.6</td>
<td>10.6$^a$</td>
</tr>
<tr>
<td>162</td>
<td><img src="image" alt="Structure" /></td>
<td>$\text{CH}_3$</td>
<td>8.2$^a$</td>
<td>5±3.4</td>
</tr>
</tbody>
</table>

$^1$Trypanosoma brucei brucei strain 427. $^a$ number of replicates (n) = 1
Table 3.10 Summary of dose-response of select GSK256066 analogs showing TbrPDEB1 IC$_{50}$ ≤ 5 µM.

<table>
<thead>
<tr>
<th>Compound</th>
<th>R$_1$</th>
<th>R$_2$</th>
<th>R$_3$</th>
<th>TbrPDEB1 (IC$_{50}$, µM)</th>
<th>Cell culture$^1$ (EC$_{50}$, µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>159</td>
<td></td>
<td></td>
<td>$\text{CH}_2\text{CH}_3$</td>
<td>3.8±0.6</td>
<td>0.63$^a$</td>
</tr>
<tr>
<td>181</td>
<td></td>
<td></td>
<td>$\text{CH}_3$</td>
<td>5.7±1.6</td>
<td>3.9±2</td>
</tr>
</tbody>
</table>

$^1$Trypanosoma brucei brucei strain 427. $^a$ number of replicates (n) = 1

Thus, it is possible that the disconnect between the biochemical TbrPDEB assay and the T. brucei cellular growth assay could be due to inhibition of other trypanosomal targets. An important limitation of this series remains the exceedingly high potency of these compounds against human PDE4 (Table 3.11), resulting in a significant challenge for achieving selectivity.$^{107}$
**Table 3.11** Potencies of GSK256066 and 2 analogs against TbrPDEB1 and hPDE4

<table>
<thead>
<tr>
<th>Compound</th>
<th>TbrPDEB1 (IC$_{50}$ µM)</th>
<th>hPDE4 (IC$_{50}$ µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSK256066</td>
<td>24.6±3.6</td>
<td>7.9x10$^{-6}$</td>
</tr>
<tr>
<td>133</td>
<td>3.8±0.4</td>
<td>8.3x10$^{-5}$</td>
</tr>
<tr>
<td>132</td>
<td>5.8±0.6</td>
<td>4.2x10$^{-5}$</td>
</tr>
</tbody>
</table>

Screening data for 133 and 132 against hPDE4 courtesy of Geert Jan Sterk, Mercachem, P.O. Box 6747, 6503 GE Nijmegen, The Netherlands.
3.4.7 Summary: GSK256066-based inhibitors of TbrPDEB1

In summary, the GSK256066 original hit and over 60 synthesized analogs were evaluated for potency against TbrPDEB1. The overall SAR of this chemotype is shown in Figure 3.13. A large number of potent GSK compounds that were identified first in the biochemical assay were later tested in assays for trypanocidal activity. The screening revealed that the GSK256066 analogs are trypanocidal in a dose-responsive manner, resulting in micromolar EC\(_{50}\) values that usually mirrored closely the IC\(_{50}\) values that were first determined against the recombinant TbrPDEB1. Furthermore, we generally think that most compounds bind in a similar way to the original GSK256066 inhibitor chemotype, but we do not exclude the possibility that some
analogs might assume a different binding pose in the TbrPDEB1 based on our current SAR. In addition, some subtle differences between the human PDE4 SAR and TbrPDEB1 were found and disclosed.

3.5 Summary of hPDE4 and hPDE5 inhibitor classes

In summary, our work using a target repurposing approach profiled a range of human PDE inhibitors\(^{85}\) against trypanosomal PDEs. This chapter in particular describes the synthetic routes and SAR of various sets of repurposed human PDE5 (tadalafil) and PDE4 (piclamilast and GSK256066) inhibitors against trypanosomal PDEs. This work shows that repurposing PDE4 inhibitors, not PDE5, to target homologous parasitic enzymes is a viable approach. Future work will need to address both the selectivity issue and the need for increased potency for the parasitic enzymes vs. the human homologs.
Chapter 4: Status & future directions for the development of anti-trypanosomal drugs
4.1 Status & future directions for repurposing existent Aur kinase inhibitors

4.1.1 Success achieved with Aur kinase inhibitors

The work described in this thesis has provided evidence that repurposing human Aurora kinase inhibitors to develop new anti-trypanosomal leads is achievable. The benchmarking data of human Aurora A and B inhibitors have shown that some of these small molecules not only inhibit TbAUK1, but also that this inhibitor class inhibits \textit{T. brucei} growth. For this project we performed a phenotypic approach that was complemented with direct TbAUK1 inhibition data for some compounds (1, 2, 5a). Our program has thus provided the launching pad for repurposing human Aurora kinase inhibitors. Using existing medicinal chemistry and structural biology information, we have designed Aurora kinase analogs that displayed cellular selectivity for trypanosomes \textit{versus} a mammalian cell line (MOLT-4). This observed selectivity profile represents proof-of-concept for the approach, substantiating one of our project goals.

We have also tried to advance our work by confirming the mechanism of action, and by elucidating what other kinases might be involved by developing clickable danusertib analogs for use as tool compounds and affinintiy probes. Unfortunately, the synthesized analogs (azide and alkyne) did not inhibit trypanosomal growth, even at high concentrations, but other tags are planned that will help address this issue.

In addition, some preliminary \textit{in vivo} studies were performed with the scaled up batches of AT-9283 and danusertib. As reported in Chapter 2, AT-9283 was able to reduce parasitemia in infected mice by almost 14 fold and also extend the life of infected mice by 36 hours when compared to the control mice. Taken as a whole, these data support the concept that Aurora
kinase inhibitors, in particular AT-9283, represent viable lead compounds that reduce parasitemia in mice. Our collaborators are currently conducting in in vivo studies with danusertib to determine its ability to reduce parasitemia in infected mice and this data will be reported in due course.

**4.1.2 Some next steps on the Auk project**

The danusertib chemotype was extensively studied in the headgroup region. A region of interest that has seen only some preliminary SAR is represented by the solvent-exposed region (Figure 4.1). A methodical reduction of the size of the solvent-exposed region should be undertaken. This reduction of molecular weight and improvement in physicochemical properties may help attain CNS permeation (see below), and will also complete the SAR evaluation of the danusertib chemotype. What is more, once the optimal fragment for activity is determined for this region, other possible clickable analogs compounds could be designed. Some proposed analogs to help achieve the goals highlighted above are disclosed in Figure 4.1.

In addition, the exploration of other Aurora kinase inhibitor chemotypes should be undertaken. For example, ZM-447439 (Figure 4.2), an Aurora B inhibitor was shown to inhibit the cell growth of trypanosomes with an EC$_{50}$ = 200 nM whereas the inhibition of TbAUK1 in the biochemical assay was approximated to have an IC$_{50}$ <10 µM. This tells us that some Aurora kinase inhibitors might achieve growth inhibition of trypanosomes cell cultures via other target(s) besides TbAUK1. The synthesis of a ZM-447439 analog that could be used for affinity chromatography (Figure 4.2) may help elucidate these targets and potentially reveal another
Figure 4.1 Some analogs proposed for synthesis for the solvent-exposed region.

Figure 4.2 Structure of proposed ZM-447439 TAG.
enzyme that is essential to trypanosomes.

Another eventual goal of this project will be to evaluate the ability of danusertib analogs to penetrate the CNS. This is important because the lethal stage of HAT is when the parasite invades the CNS, and thus any new drugs should require brain exposure to have an effect on the parasite in the second stage of the disease. While the best way to assess CNS penetration is via in vivo experimentation, this is costly and time-consuming. An alternate approach to evaluate brain permeability is to look at how various metrics correlate with CNS penetration. For example, Wager et al. have published a desirable CNS multiparameter optimization (MPO) that is intended to aid researchers when analyzing their lead molecules as CNS drugs.¹¹¹ This tool (CNS MPO) was validated using 119 marketed CNS drugs and 108 Pfizer CNS candidates, and the results obtained indicate that 74% of the marketed CNS drugs had a high MPO score ≥ 4 (using a scale of 0-6). This algorithm is a useful tool to identify with a higher success rate CNS permeable compounds since it provides greater flexibility in CNS compound design because it does not provide a single parameter or strict cutoffs. Their work¹¹¹ focuses on a set of six physicochemical parameters:

a. lipophilicity, calculated partition coefficient (ClogP);
b. calculated distribution coefficient at pH = 7.4 (ClogD);
c. molecular weight (MW);
d. topological polar surface area (TPSA);
e. number of hydrogen bond donors (HBD);
f. most basic center (pKa)
One of our lead molecules 8 was analyzed using this published desirable CNS MPO, and this is shown in Table 4.1. If we use this CNS MPO to evaluate our lead compound then we observe that the properties of our drug are just outside range of CNS-active drugs. Thus, one future goal is to work on reducing the MW, ClogP and HBD.

Table 4.1 CNS MPO of a lead danusertib analog

<table>
<thead>
<tr>
<th>Property</th>
<th>CNS MPO</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>MW</td>
<td>494.6</td>
<td>≤ 360</td>
</tr>
<tr>
<td>ClogP</td>
<td>3.5</td>
<td>≤ 3</td>
</tr>
<tr>
<td>ClogD</td>
<td>3.48</td>
<td>≤ 2</td>
</tr>
<tr>
<td>HBD (OH+NH)</td>
<td>2</td>
<td>≤ 0.5</td>
</tr>
<tr>
<td>TPSA</td>
<td>84.6</td>
<td>40 &lt; x ≤ 90</td>
</tr>
<tr>
<td>pKa</td>
<td>8.12</td>
<td>≤ 8</td>
</tr>
</tbody>
</table>

MPO Score 3.5
4.2 Status and future directions for repurposing existent PDE inhibitors

4.2.1 What we have learned thus far from repurposing PDE inhibitors

The work presented in Chapter 1 and 3 has shown that trypanosomal PDEs are a viable target for an anti-parasitic approach. This work has identified micromolar potent compounds based on two hPDE4 chemotypes piclamilast and GSK256066. Furthermore, it was shown that the GSK256066 template is still very potent against hPDE4, thus the selectivity challenge remained. We are currently working to determine if the differences between the parasitic enzyme TbrPDEB1 and human PDE4 are sufficient to achieve selectivity, particularly with the GSK256066 chemotype. We also acknowledge that a different binding mode could be possible for both the piclamilast and GSK256066 chemotypes.

4.2.2 Other approaches

The PDE project has relied thus far on repurposing human PDE inhibitors to find new leads against the parasitic enzymes, TbrPDEB1 and 2. Given the flat SAR and the selectivity challenges observed for the investigated chemotypes we have considered other approaches. First, we would like to know how our compounds bind to the parasitic enzymes. The use of homology modeling to drive our drug design was a helpful tool to help explain some of the changes in activity that we have observed. However, the differences between the parasitic enzymes and the human enzymes seem to be indeed very subtle, and some key information might be overlooked when using the homology modeling to aid our drug design. Therefore, our lab has initiated an international collaboration with Dr. Raymond Hui from the Structural
Genomics Consortium in Canada. Dr. Hui's research group will try to elucidate the crystal structure of the parasitic enzyme bound with our lead compounds. To facilitate his efforts our group has sent a large set of TbrPDEB1 inhibitors. A set of GSK256066 analogs (Figure 4.3) was selected for co-crystallization, as these have raised specific questions regarding likely binding conformations. For example, as highlighted in Chapter 3, we believe that some compounds might not be binding as we originally thought (e.g. compound 132) since compounds like 171, 174 and 182 retain their activity against the parasitic enzyme, despite the removal of putative metal-binding functionality.

![GSK analogs selected for X-ray crystallography.](image)

Furthermore, other possible approaches that could be used on this project are a Fragment based approach or an HTS screening. Both strategies would ideally provide new lead
chemotypes that upon optimization will result in more potent and selective compounds. The HTS screening is a more costly approach and this could perhaps be achieved *via* an industrial collaboration. Thus, despite our current challenges in establishing potent and selective TbrPDE inhibitors, we remain optimistic that a combination of these approaches will bear fruit.
APPENDIX 1

Supplemental Synthetic Methods and Characterization for Chapter 2
Experimental procedures and characterization data for Chapter 2 compounds

Reagents were obtained from Sigma-Aldrich, Inc. (St. Louis, MO), Fisher Scientific, Inc. (Pittsburgh, PA), Frontier Scientific, Inc. (Logan, Utah), Tokyo Chemical Industry Co. (TCI), Ltd. (Montgomeryville, PA), Chem-Impex International, Inc. (Wood Dale, IL), Synthonix (Wake Forest, NC) and used as received. Reaction solvents were purified by passage through alumina columns on a purification system manufactured by Innovative Technology (Newburyport, MA). The Biotage Isolera flash purification system used has the following specifications: solvent delivery (Two constant volume, 3-mL, electric HPFC pumps), flow rate (1 – 200 mL/min), pressure limit (145 psi), UV detection (choice of variable wavelength 200–400 nm, fixed 254 nm, or UV-VIS 200–800 nm detectors), UV collection modes (single/dual/λ-All wavelengths), fractionation modes (volume, threshold, threshold with volume, low slope, medium slope, custom slope), collection vessels (test tubes, 13, 16, 18, and 25 mm), power (100 – 240 VAC, 50/60 Hz, 4.0 A), system control and data management (on-board computer with 10.4” diagonal touch-screen interface). NMR spectra were obtained on Varian NMR systems, operating at 400 MHz or 500 MHz for ¹H acquisitions. LCMS analysis was performed using a Waters Alliance reverse-phase HPLC, with single-wavelength UV-visible detector and LCT Premier time-of-flight mass spectrometer (electrospray ionization). Specific rotations $\alpha_D$ were obtained at 21°C using the P-2000 Digital Polarimeter (from Jasco Corporation) in the solvent specified.

Analytical Chemistry: Preparative Method for Final Compounds Purification Instruments:
Instruments: Waters 2525 binary pump, Waters 2767 sample manager, Waters 2489 UV/Visible detector, Waters 2x 515 pumps, Waters pump control, Waters column fluidics organizer, MicroMass ZQ mass detector

Detection: UV: @254 nm, Mass: ESI + mode, set to the mass of interest compounds

Collection: Mass and UV combination

Column: Waters Symmetry C8 30x50mm, 5μm or Waters Xbridge OBD RP18 30x50mm, 5μm

Mobile Phase: A: 0.1% v/v Formic acid in Water  B: Acetonitrile

Typical Preparative HPLC method (start gradient may vary based on compound’s retention time on the analytical system), and the method run time was 8 min.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Flow rate</th>
<th>A%</th>
<th>B%</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>40 ml/min</td>
<td>95%</td>
<td>5%</td>
</tr>
<tr>
<td>7.50</td>
<td>40 ml/min</td>
<td>0%</td>
<td>100%</td>
</tr>
<tr>
<td>7.90</td>
<td>40 ml/min</td>
<td>0%</td>
<td>100%</td>
</tr>
<tr>
<td>7.91</td>
<td>40 ml/min</td>
<td>95%</td>
<td>5%</td>
</tr>
<tr>
<td>8.00</td>
<td>40 ml/min</td>
<td>95%</td>
<td>5%</td>
</tr>
</tbody>
</table>

Analytical Chemistry: Analytical Method for Compounds purity determination

Instruments: Waters e2795 Alliance HPLC separation module, Waters 2489 UV/visible detector, Waters LCT premier micromass

Detection: UV: @254 nm, Mass: ESI + mode, scan mass range 100-1000

Columns: Waters SunFire C18 4.6x50mm, 3.5μm or Waters SunFire C8 4.6x50mm, 3.5μm

Mobile Phase: A: 0.1% v/v Formic acid in Water  B: Acetonitrile, Method Run time: 4 min

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Flow rate</th>
<th>A%</th>
<th>B%</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>2 ml/min</td>
<td>95%</td>
<td>5%</td>
</tr>
<tr>
<td>3.75</td>
<td>2 ml/min</td>
<td>0%</td>
<td>100%</td>
</tr>
<tr>
<td>3.85</td>
<td>2 ml/min</td>
<td>0%</td>
<td>100%</td>
</tr>
<tr>
<td>3.95</td>
<td>2 ml/min</td>
<td>95%</td>
<td>5%</td>
</tr>
<tr>
<td>4.00</td>
<td>2 ml/min</td>
<td>95%</td>
<td>5%</td>
</tr>
</tbody>
</table>
All newly synthesized compounds were deemed >95% pure by LCMS analysis prior to submission for biological testing. Published methods were employed for preparation of danusertib (1)\(^{112}\), PHA-680632 (2)\(^{113}\), 3\(^{113}\), 5b\(^{113}\), 46a\(^{113}\) and 51a\(^{97}\).

Ethyl5-benzoyl-3-(4-(4-methylpiperazin-1-yl)benzamido)-5,6-dihydropyrrolo[3,4c]pyrazole-1 (4H)-carboxylate (4a). To 0.015 g of 3 (0.034 mmol) was added pyridine (2.3 ml, 29.1 mmol) and benzoyl chloride (7.95 µL, 0.069 mmol). The reaction was stirred for 16 hours.
The reaction was concentrated and the crude product was purified via silica gel chromatography, eluting with 0-8% MeOH in DCM to give 4a (Yield: 21%).  

$^1$H NMR (500 MHz, CD$_3$OD) δ 7.89 and 7.78 (2d, $J = 8.8$ Hz, 2H, rotamers), 7.59 - 7.32 (m, 2H), 7.49 - 7.55 (m, 3H), 7.06 and 6.95 (2d, $J = 8.8$ Hz, 2H, rotamers), 4.95 - 4.82 (m, 4H), 4.50 (q, $J = 7.0$ Hz, 2H), 3.34 - 3.41 (m, 4H), 2.57 - 2.64 (m, 4H), 2.32 and 2.38 (2s, 3H, rotamers), 1.27 and 1.27 (2t, $J = 7.3$, 9.3 Hz, 3H, rotamers). LCMS found 503.01, [M+H]$^+$.

N-(5-Benzoyl-1,4,5,6-tetrahydropyrrolo[3,4-c]pyrazol-3-yl)-4-(4-methylpiperazin-1-yl)benzamide (5a). A solution of 4a (5.80 mg, 0.012 mmol) in MeOH (3 mL) and Et$_3$N (0.3 mL) was stirred at 30 °C for 4 hours. The reaction was concentrated. The residue was dissolved twice in diethyl ether and concentrated to produce a white solid, which was triturated with a mixture of EtOAc (3 mL) and ether (0.3 mL) to give 5a (Yield: 99%).  

$^1$H NMR (500 MHz, DMSO-$d_6$) δ 7.82 (7.91 minor) (d, $J = 8.5$ Hz, 2H), 7.6-7.54 (m, 2H), 7.49-7.45 (m, 3H), 6.92 (6.98 minor) (d, $J = 9.0$ Hz, 2H) 4.69-4.52 (m, 4H), 3.25-3.23 (3.29-3.27 minor) (m, 4H), 2.41-2.39 (2.44-2.43 minor) (m, 4H), 2.20 (2.21 minor) (s, 3H). The sets of amide rotamer signals that...
were doubled at room temperature and listed as minor were shown to coalesce at 140 °C. LCMS found 431.01, [M+H]+.

Ethyl 3-(4-(4-methylpiperazin-1-yl)benzamido)-5-tosyl-5,6-dihydropyrrolo[3,4-c]pyrazole-1(4H)-carboxylate (4c). To 0.013 g of 3 (0.029 mmol) was added pyridine (2 mL), and then tosyl chloride (5.5 mg, 0.03 mmol). The reaction mixture was stirred at rt for 16 h. The solvent was concentrated and the crude product was purified via silica gel chromatography, eluting with 0-10% MeOH in DCM to give 4c (Yield: 23%). ¹H NMR (500 MHz, CD₃OD)

δ 7.83 (d, J = 8.5 Hz, 2H), 7.80 (d, J = 8.7 Hz, 2H), 7.42 (d, J = 8.5 Hz, 2H), 7.00 (d, J = 8.7 Hz, 2H), 4.65-4.63 (m, 4H), 4.44 (q, 2H), 3.39-3.37 (m, 4H), 2.62-2.60 (m, 4H), 2.41 (s, 3H), 2.36 (s, 3H), 1.41 (t, J = 7.2 Hz, 3H). LCMS found 553.01, [M+H]+.
4-(4-Methylpiperazin-1-yl)-N-(5-tosyl-1,4,5,6-tetrahydropyrrolo[3,4-c]pyrazol-3-yl)benzamide (5c). A solution of 4c (3.7 mg, 6.7 µmol) in MeOH (1 mL) and Et$_3$N (0.1 mL) was stirred at 30 °C for 4 hours. The solvent was concentrated. The residue was dissolved twice in diethyl ether and concentrated to produce a white solid, which was triturated with a mixture of EtOAc (3 mL) and ether (0.3 mL) to give 5c (Yield: 93%). $^1$H NMR (500 MHz, CD$_3$OD) δ 7.83 (d, J = 9.0 Hz, 2H), 7.88 (d, J = 8.5 Hz, 2H), 7.41 (d, J = 8.5 Hz, 2H), 7.01 (d, J = 9.0 Hz, 2H), 4.62-4.35 (m, 4H), 3.39-3.37 (m, 4H), 2.64-2.62 (m, 4H), 2.41 (s, 3H), 2.37 (s, 3H). LCMS found 481.01, [M+H]$^+$.

**General procedure A**

**Library synthesis.** To the various carboxylic acids (obtained from Frontier Scientific, Inc., 0.076 mmol, 1 equiv.) was added dry DCM (0.5 mL), oxalyl chloride (0.076 mmol, 1 equiv.),
and then 2 drops of DMF. The reactions were placed in the shaker and reacted overnight at ambient temperature for 24 hours. A solution of the hydrochloride salt of 3 (0.048 mmol, 1 equiv.) in 1.5 mL of DMF was added, followed by DIEA (0.240 mmol, 5 equiv.). The vials were agitated in the shaker for 24 hours at ambient temperature. The solvent was removed in a Genevac HT24 centrifugal evaporator, and the crude intermediates were carried forward without purification. To each of the above crude products was added 10% TEA in MeOH (2 mL). The reaction vials were then placed in the shaker at 33 °C overnight, and the solvent was evaporated. All the crude products were purified via preparative HPLC to >95% purity to give the desired products in moderate to good yields as described below.
4-(4-Methylpiperazin-1-yl)-N-(5-(2-(naphthalen-1-yl)acetyl)-1,4,5,6-tetrahydropyrrolo[3,4-c]pyrazol-3-yl)benzamide (8), (Yield: 30%). $^1$H NMR (500 MHz, DMSO-$d_6$) $\delta$ 10.65 (s, 1H), 7.90-7.84 (m, 5H), 7.77 (d, $J = 8.5$ Hz, 1H), 7.48-7.43 (m, 3H), 6.99 (d, $J = 9.5$ Hz, 2H), 4.80-4.47 (m, 4H), 4.10-4.02 (m, 2H), 3.92 (d, $J = 10.5$ Hz, 2H), 3.15 (m, 2H), 2.56 (m, 4H), 2.31 (s, 3H). $^{13}$C NMR (126 MHz, DMSO-$d_6$) $\delta$ 169.5, 169.5, 133.3, 133.0, 131.8, 129.3, 128.2, 127.7, 127.5, 127.4, 126.0, 125.5, 113.5, 53.9, 48.6, 46.3, 42.1, 40.1. LCMS found 495.01, [M+H]$^+$. 

4-(4-Methylpiperazin-1-yl)-N-(5-(2-(2,3,6-trifluorophenyl)acetyl)-1,4,5,6-tetrahydropyrrolo[3,4-c]pyrazol-3-yl)benzamide (9), (Yield: 62%). $^1$H NMR (400 MHz, DMSO-$d_6$) $\delta$ 10.70 (s, 1H), 7.93-7.89 (m, 2H), 7.50-7.38 (m, 1H), 7.18-7.10 (m, 1H), 7.01 (d, $J = 8.4$ Hz, 2H), 6.56 (s, 1H), 4.86-4.45 (m, 4H), 3.87 (s, 3H), 3.40-3.30 (m, 4H), 2.70-2.63 (m, 4H), 2.38 (s, 3H). LCMS found 499.01, [M+H]$^+$. 

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N-(5-(2-Methoxy-2-phenylacetyl)-1,4,5,6-tetrahydropyrrolo[3,4-c]pyrazol-3-yl)-4-(4-methylpiperazin-1-yl)benzamide (10), (Yield: 81%). $^1$H NMR (400 MHz, DMSO-$d_6$) $\delta$ 10.67 (d, J = 9.6 Hz, 1H), 7.89 (d, J = 12.0 Hz, 2H), 7.44-7.32 (m, 5H), 7.01 (d, J = 12.0 Hz, 2H), 5.10 (d, J = 12.0 Hz, 1H), 4.82-4.39 (m, 4H), 3.40-3.35 (m, 4H), 3.31 (d, J = 3.2 Hz, 3H), 2.80-2.65 (m, 4H), 2.48 (s, 3H). LCMS found 475.01, [M+H]$^+$.  

N-(5-(2-(3-Chlorophenyl)acetyl)-1,4,5,6-tetrahydropyrrolo[3,4-c]pyrazol-3-yl)-4-(4-methylpiperazin-1-yl)benzamide (11), (Yield: 27%). $^1$H NMR (500 MHz, DMSO-$d_6$) $\delta$ 10.70 (s, 1H), 7.92-7.89 (m, 2H), 7.35-7.21 (m, 4H), 7.01 (d, J = 9.0 Hz, 2H), 4.76-4.44 (m, 4H), 3.76 (d, J = 14.0 Hz, 2H), 3.37 (m, 4H), 2.72 (m, 4H), 2.42 (s, 3H). LCMS found 479.01, [M+H]$^+$. 

\[ \text{N-(5-(2-(3-Chlorophenyl)acetyl)-1,4,5,6-tetrahydropyrrolo[3,4-c]pyrazol-3-yl)-4-(4-methylpiperazin-1-yl)benzamide (11), (Yield: 27%).} \]
N-(5-(3-Methyl-2-phenylbutanoyl)-1,4,5,6-tetrahydropyrrolo[3,4-c]pyrazol-3-yl)-4-(4-methylpiperazin-1-yl)benzamide (12), (Yield: 67%). $^1$H NMR (500 MHz, CD$_3$OD) δ 7.90-7.86 (m, 2H), 7.46-7.40 (m, 2H), 7.35-7.30 (m, 2H), 7.28-7.22 (m, 1H), 7.04 (t, J = 8.5 Hz, 2H), 5.03 (d, J = 12.0 Hz, 1H), 4.95-4.44 (m, 4H), 3.50-3.40 (m, 4H), 2.86-2.81 (m, 4H), 2.53 (s, 3H), 2.46-2.40 (m, 1H), 1.08 (d, J = 7.0 Hz, 3H), 0.71 (t, J = 4.5Hz, 3H). $^{13}$C NMR (126 MHz, DMSO-$d_6$) δ 172.4, 172.4, 153.6, 139.6, 139.6, 130.0, 129.9, 129.2, 129.1, 129.1, 127.5, 114.2, 57.4, 57.1, 54.6, 46.9, 45.6, 32.6, 32.4, 22.2, 22.1. LCMS found 487.01, [M+H]$^+$.  

![13](NEU-343)

4-(4-Methylpiperazin-1-yl)-N-(5-(2-(p-tolyl)propanoyl)-1,4,5,6-tetrahydropyrrolo[3,4-c]pyrazol-3-yl)benzamide (13), (Yield: 68%). $^1$H NMR (400 MHz, DMSO-$d_6$) δ 10.62 (d, J = 9.6 Hz, 1H), 7.89-7.85 (m, 2H), 7.21-7.17 (m, 2H), 7.13-7.10 (m, 2H), 7.70-6.97 (m, 2H), 4.82-4.77 (m, 1H), 4.48-3.88 (m, 4H), 3.40-3.30 (m, 4H), 2.61-2.55 (m, 4H), 2.32 (s, 3H), 2.23 (s, 3H), 1.30 (d, J = 6.8 Hz, 3H). LCMS found 473.01, [M+H]$^+$.  

![14](NEU-340)
N-(5-(2-(3,5-Dimethylphenyl)acetyl)-1,4,5,6-tetrahydropyrrolo[3,4-c]pyrazol-3-yl)-4-(4-methylpiperazin-1-yl)benzamide (14), (Yield: 75%).\textsuperscript{1}H NMR (400 MHz, DMSO-\textit{d}_6) \delta 10.69 (s, 1H), 7.88 (d, J = 8.4 Hz, 2H), 6.98 (d, J = 8.4 Hz, 2H), 6.87-6.84 (m, 3H), 4.72-4.43 (m, 4H), 3.62 (d, J = 8.0 Hz, 2H), 2.53-2.48 (m, 4H), 2.27 (s, 3H), 2.23 (s, 6H). LCMS found 473.01, [M+H]\textsuperscript{+}.

\[\text{15} \quad \text{(NEU-334)}\]

N-(5-(2-(2,5-Dimethylphenyl)acetyl)-1,4,5,6-tetrahydropyrrolo[3,4-c]pyrazol-3-yl)-4-(4-methylpiperazin-1-yl)benzamide (15), (Yield: 74%).\textsuperscript{1}H NMR (400 MHz, DMSO-\textit{d}_6) \delta 10.70 (s, 1H), 7.90 (d, J = 8.8 Hz, 2H), 7.04-6.99 (m, 3H), 6.94-6.92 (m, 2H), 6.56 (s, 1H), 4.76-4.46 (m, 4H), 3.66 (d, J = 4.0 Hz, 2H), 3.40-3.30 (m, 4H), 2.70-2.60 (m, 4H), 2.38 (s, 3H), 2.22 (s, 3H), 2.16 (s, 3H). LCMS found 473.01, [M+H]\textsuperscript{+}.

\[\text{16} \quad \text{(NEU-341)}\]

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4-(4-Methylpiperazin-1-yl)-N-(5-(2-(2,3,5-trifluorophenyl)acetyl)-1,4,5,6-tetrahydropyrrolo[3,4-c]pyrazol-3-yl)benzamide (16), (Yield: 73%). $^1$H NMR (500 MHz, CD$_3$OD) $\delta$ 7.89-7.86 (m, 2H), 7.12-7.07 (m, 1H), 7.05-7.02 (m, 2H), 7.0-6.95 (m, 1H), 4.93-4.59 (m, 4H), 3.91 (d, J = 5.5 Hz, 2H), 3.42-3.40 (m, 4H), 2.77-2.73 (m, 4H), 2.46 (s, 3H). LCMS found 499.01, [M+H]$^+$. 

N-(5-(2-(2-Methyl-1H-indol-3-yl)acetyl)-1,4,5,6-tetrahydropyrrolo[3,4-c]pyrazol-3-yl)-4-(4-methylpiperazin-1-yl)benzamide (17), (Yield: 70%). $^1$H NMR (400 MHz, DMSO-$d_6$) $\delta$ 10.84 (d, J = 8.0 Hz, 1H), 10.68 (d, J = 6.4 Hz, 1H), 7.89 (m, 2H), 7.46 (d, J = 8.0 Hz, 1H), 7.23-7.20 (m, 1H), 7.01-6.88 (m, 4H), 4.76-4.41 (m, 4H), 3.71 (s, 2H), 3.33-3.30 (m, 4H), 2.61-2.56 (m, 4H), 2.36 (d, J = 16.0 Hz, 3H), 2.31 (s, 3H). LCMS found 498.01, [M+H]$^+$. 

18 (NEU-333)
N-(5-(2-(3,5-Difluorophenyl)acetyl)-1,4,5,6-tetrahydropyrrolo[3,4-c]pyrazol-3-yl)-4-(4-methylpiperazin-1-yl)benzamide (18), (Yield: 56%). $^1$H NMR (500 MHz, CD$_3$OD) $\delta$ 7.89 (d, J = 9.0 Hz, 2H), 7.07-7.05 (m, 2H), 6.96-6.93 (m, 2H), 6.86-6.82 (m, 1H), 4.85-4.56 (m, 4H), 3.82 (s, 2H), 3.54-3.52 (m, 4H), 3.17-3.13 (m, 4H), 2.75 (s, 3H). LCMS found 481.01, [M+H]$^+$.

N-(5-(2-(2,4-Difluorophenyl)acetyl)-1,4,5,6-tetrahydropyrrolo[3,4-c]pyrazol-3-yl)-4-(4-methylpiperazin-1-yl)benzamide (19), (Yield: 33%). $^1$H NMR (400 MHz, DMSO-d$_6$) $\delta$ 10.70 (s, 1H), 7.93-7.89 (m, 2H), 7.37-7.19 (m, 2H), 7.05-7.00 (m, 3H), 6.56 (s, 1H), 4.80-4.44 (m, 4H), 3.76 (d, J = 4.8 Hz, 2H), 3.42-3.29 (m, 4H), 2.75-2.65 (m, 4H), 2.42 (s, 3H). LCMS found 481.01, [M+H]$^+$.
N-(5-(2-(2,5-Difluorophenyl)acetyl)-1,4,5,6-tetrahydropyrrolo[3,4-c]pyrazol-3-yl)-4-(4-methylpiperazin-1-yl)benzamide (20), (Yield:18%). $^1$H NMR (500 MHz, CD$_3$OD) $\delta$ 7.92-7.90 (m, 2H), 7.15-7.02 (m, 5H), 4.93-4.59 (m, 4H), 3.85 (d, $J$ = 6.0 Hz, 2H), 3.56-3.54 (m, 4H), 3.23-3.19 (m, 4H), 2.81 (s, 3H). LCMS found 481.01, [M+H]$^+$.

![Chemical Structure of 20](image)

N-(5-(2-(2,6-Difluorophenyl)acetyl)-1,4,5,6-tetrahydropyrrolo[3,4-c]pyrazol-3-yl)-4-(4-methylpiperazin-1-yl)benzamide (21), (Yield: 49%). $^1$H NMR (400 MHz, DMSO-$d_6$) $\delta$ 10.70 (s, 1H), 7.91 (t, $J$ = 8.0 Hz, 2H), 7.40-7.32 (m, 1H), 7.10-7.06 (m, 2H), 7.02 (d, $J$ = 8.0 Hz, 2H), 6.56 (s, 1H), 4.85-4.45 (m, 4H), 3.79 (s, 2H), 3.40-3.30 (m, 4H), 2.80-2.60 (m, 4H), 2.48 (s, 3H). LCMS found 481.01, [M+H]$^+$.

![Chemical Structure of 21](image)
N-(5-(2-(3,4-Difluorophenyl)acetyl)-1,4,5,6-tetrahydropyrrolo[3,4-c]pyrazol-3-yl)-4-(4-methylpiperazin-1-yl)benzamide (22), (Yield:48%). $^1$H NMR (500 MHz, CD$_3$OD) $\delta$ 7.91-7.88 (m, 2H), 7.27-7.19 (m, 2H), 7.15-7.11 (m, 1H), 7.10-7.07 (m, 2H), 4.88-4.59 (m, 4H), 3.80 (d, J = 5.0 Hz, 2H), 3.55-4.9 (m, 4H), 3.17-3.11 (m, 4H), 2.74 (d, J = 6.0 Hz, 3H). LCMS found 481.01, [M+H]$^+$. 

![Chemical structure of 22](image)

4-(4-Methylpiperazin-1-yl)-N-(5-(2-(2,4,6-trifluorophenyl)acetyl)-1,4,5,6-tetrahydropyrrolo[3,4-c]pyrazol-3-yl)benzamide (23), (Yield:54%). $^1$H NMR (400 MHz, DMSO-$d_6$) $\delta$ 10.70 (s, 1H), 7.72-7.89 (m, 2H), 7.20-7.16 (m, 2H), 7.00 (d, J = 8.8 Hz, 2H), 6.56 (s, 1H), 4.84-4.45 (m, 4H), 3.77 (s, 2H), 3.40-3.30 (m, 4H), 2.70-2.60 (m, 4H), 2.36 (s, 3H). LCMS found 499.01, [M+H]$^+$. 

![Chemical structure of 23](image)
4-(4-Methylpiperazin-1-yl)-N-(5-(3-oxo-2,3-dihydro-1H-indene-1-carbonyl)-1,4,5,6-tetrahydropyrrolo[3,4-c]pyrazol-3-yl)benzamide (24), (Yield: 18%). ¹H NMR (500 MHz, CD3OD) δ 7.92-7.88 (m, 2H), 7.76 (d, J = 8.0 Hz, 1H), 7.69 (t, J = 7.5 Hz, 1H), 7.61 (d, J = 8.0 Hz, 1H), 7.50 (t, J = 7.5 Hz, 1H), 7.10-7.06 (m, 2H), 5.29-5.04 (m, 2H), 4.76-4.72 (m, 1H), 4.64-4.50 (m, 2H), 3.57-3.45 (m, 4H), 3.12-3.07 (m, 4H), 3.02-3.00 (m, 1H), 2.92-2.84 (m, 1H), 2.71 (d, J = 7.5 Hz, 3H). LCMS found 485.01, [M+H]+.

N-(5-(2-(2,4-Dimethylthiazol-5-yl)acetyl)-1,4,5,6-tetrahydropyrrolo[3,4-c]pyrazol-3-yl)-4-(4-methylpiperazin-1-yl)benzamide (25), (Yield: 27%). ¹H NMR (500 MHz, DMSO-d6) δ 10.68 (s, 1H), 7.92-7.89 (m, 2H), 7.00 (d, J = 8.5 Hz, 2H), 4.75 (d, J = 23.5 Hz, 2H), 4.46 (d, J =
23.5 Hz, 2H), 3.86 (d, J = 9.5 Hz, 2H), 3.36-3.33 (m, 4H), 2.68-2.63 (m, 4H), 2.52 (s, 3H), 2.36 (s, 3H), 2.24 (d, J = 6.5 Hz, 3H). LCMS found 480.1 [(M+H)]^+.

![Image](image1.png)

4-(4-Methylpiperazin-1-yl)-N-(5-(2-(2-methylthiazol-4-yl)acetyl)-1,4,5,6-tetrahydropyrrolo[3,4-c]pyrazol-3-yl)benzamide (NEU-26), (Yield: 65%). ^1H NMR (400 MHz, DMSO-d6) δ 10.71 (d, J = 7.6 Hz, 1H), 7.92 (d, J = 8.6 Hz, 2H), 7.24 (d, J = 7.2 Hz, 1H), 7.01 (d, J = 8.6 Hz, 2H), 4.78 (d, J = 15.0 Hz, 2H), 4.45 (d, J = 15.0 Hz, 2H), 3.79 (s, 2H), 3.43-3.36 (m, 4H), 2.78-2.72 (m, 4H), 2.60 (s, 3H), 2.43 (s, 3H). LCMS found 466.01, [M+H]^+.

![Image](image2.png)

N-(5-(3-methyl-2-phenylpentanoyl)-1,4,5,6-tetrahydropyrrolo[3,4-c]pyrazol-3-yl)-4-(4-methylpiperazin-1-yl)benzamide (27), (Yield: 27%). ^1H NMR (500 MHz, DMSO-d6) δ 10.60 (br. s., 1H), 7.87 and 7.90 (2d, J = 8.3 Hz, 2H, rotamers), 7.35 - 7.43 (m, 2H), 7.31 (t, J = 7.5 Hz, 2H), 7.19 - 7.25 (m, 1H), 6.94 - 7.02 (m, 2H), 4.90 (m, 1H), 4.36-4.65 (m, 3H), 3.49 (t, J =
10.9 Hz, 1H), 3.26 - 3.29 (m, 4H), 2.40 - 2.45 (m, 4H), 2.20 and 2.21 (2s, 3H, rotamers), 2.08 - 2.17 (m, 1H), 1.51-1.62 (m, 1H), 1.15-1.39 (m, 1H), 0.96 (d, J = 6.3 Hz, 1H), 0.88 (m, 2H), 0.58 and 0.73 (2t, J = 7.2 Hz, 3H, rotamers). LCMS found 501.01, [M+H]+.

![Chemical Structure](image)

N-(5-(5-methyl-2-(o-tolyl)hexanoyl)-1,4,5,6-tetrahydropyrrolo[3,4-c]pyrazol-3-yl)-4-(4-methylpiperazin-1-yl)benzamide (28), (Yield: 30%). 1H NMR (500 MHz, CD3OD) δ7.83 and 7.86 (2d, J = 8.7 Hz, 2H, rotamers), 7.19 - 7.30 (m, 2H), 7.11 - 7.18 (m, 2H), 7.01 and 7.03 (2d, J = 8.7 Hz, 2H, rotamers), 4.92 (d, J = 12.7 Hz, 1H), 4.80 (d, J = 13.1 Hz, 1H), 4.28-4.76 (m, 3H), 3.94 - 4.04 (m, 1H), 3.37 - 3.43 (m, 4H), 2.71-2.73 (m, 4H), 2.50 and 2.46 (2s, 3H, rotamers), 2.44 (s, 3H), 2.02 - 2.13 (m, 1H), 1.50 - 1.65 (m, 2H), 1.36 (dd, J = 7.3, 12.2 Hz, 1H), 1.18 (dd, J = 6.3, 11.7 Hz, 1H), 0.91 (d, J = 6.8 Hz, 3H), 0.88 (d, J = 6.3 Hz, 3H). LCMS found 529.01, [M+H]+.
N-(5-(2-cyclopentyl-2-phenylacetyl)-1,4,5,6-tetrahydropyrrolo[3,4-c]pyrazol-3-yl)-4-(4-
 methylpiperazin-1-yl)benzamide (29), (Yield: 30%). $^1$H NMR (500 MHz, DMSO-$d_6$) $\delta$ 10.74
(br. s., 1H), 7.87 and 7.90 (2d, $J$ = 8.7 Hz, 2H, rotamers), 7.36 - 7.42 (m, 2H), 7.31 (t, $J$ = 7.5 Hz,
2H), 7.20 - 7.25 (m, 1H), 6.95 - 7.01 (m, 2H), 4.83 - 4.96 (m, 1H), 4.31 - 4.58 (m, 4H), 3.49 -
3.52 (m, 1H), 3.26 - 3.30 (m, 4H), 2.43 (m, 4H), 2.20 and 2.21 (2s, 3H, rotamers), 2.09 - 2.17
(m, 1H), 1.48 - 1.61 (m, 1H), 1.04 - 1.24 (m, 1H), 0.95 (d, $J$ = 6.3 Hz, 1H), 0.88 (dt, $J$ = 3.4, 7.3
Hz, 2H), 0.71 - 0.76 (m, 1H), 0.57 - 0.61 (m, 1H). LCMS 513.01, [M+H]$^+$.  

4-(4-methylpiperazin-1-yl)-N-(5-(2-phenylpropanoyl)-1,4,5,6-tetrahydropyrrolo[3,4-
c]pyrazol-3-yl)benzamide (30), (Yield: 44%). $^1$H NMR (500 MHz, DMSO-$d_6$) $\delta$ 10.61 and
10.63 (2br. s., 1H, rotamers), 7.87 (t, \( J = 7.8 \) Hz, 2H), 7.32 (m, 4H), 7.22 (m, 1H), 6.97 and 6.98 (2d, 8.5 Hz, 2H, rotamers), 4.82 (t, \( J = 12.2 \) Hz, 1H), 4.16 - 4.53 (m, 3H), 3.97 (m, 1H), 3.28 (m, 4H), 2.42 - 2.46 (m, 4H), 2.22 (s, 3H), 1.34 (d, \( J = 6.8 \) Hz, 3H). LCMS 459.01, \([M+H]^+\).

![Chemical structure](image)

**N-(5-(2-methyl-2-phenylpropanoyl)-1,4,5,6-tetrahydropyrrolo[3,4-c]pyrazol-3-yl)-4-(4-methylpiperazin-1-yl)benzamide** (31), (Yield: 24%). \(^1\)H NMR (500 MHz, CD\(_3\)OD) \( \delta \) 7.77 and 7.87 (2d, \( J = 8.7 \) Hz, 2H, rotamers), 7.31 - 7.42 (m, 4H), 7.22 - 7.30 (m, 1H), 7.01 and 7.04 (2d, \( J = 8.7 \) Hz, 2H, rotamers), 4.61-4.71 (m, 2H), 3.88-4.07 (m, 2H), 3.39 - 3.48 (m, 4H), 2.79 - 2.88 (m, 4H), 2.53 (2s, 3H, rotamers), 1.61 (2s, 6H, rotamers). LCMS 473.01, \([M+H]^+\).

![Chemical structure](image)

**4-(4-methylpiperazin-1-yl)-N-(5-(2-phenylbutanoyl)-1,4,5,6-tetrahydropyrrolo[3,4-c]pyrazol-3-yl)benzamide** (32), (Yield: 56%). \(^1\)H NMR (500 MHz, CD\(_3\)OD) \( \delta \) 7.82 - 7.89 (m, 5H), 7.24 - 7.30 (m, 2H), 6.90 - 6.94 (m, 2H, rotamers), 4.77 - 4.84 (m, 1H), 3.86 - 4.08 (m, 2H), 3.39 - 3.48 (m, 4H), 2.80 - 2.86 (m, 4H), 2.53 (2s, 3H, rotamers), 1.62 (2s, 6H, rotamers). LCMS 471.01, \([M+H]^+\).
2H), 7.40 (d, J = 7.3 Hz, 1H), 7.30 - 7.38 (m, 3H), 7.21 - 7.28 (m, 1H), 6.98 - 7.05 (m, 2H), 4.96 (d, J = 12.2 Hz, 1H), 4.36 - 4.84 (m, 3H), 3.67 - 3.79 (m, 1H), 3.42 (m, 4H), 2.80 - 2.87 (m, 4H), 2.53 (s, 3H), 2.08 - 2.14 (m, 1H), 1.70 - 1.83 (m, 1H), 0.92 (t, J = 7.3 Hz, 3H). LCMS 473.01, [M+H]+.

4-(4-methylpiperazin-1-yl)-N-(5-(1-phenylcyclopentanecarbonyl)-1,4,5,6-tetrahydro pyrrolo[3,4-c]pyrazol-3-yl)benzamide, formate salt (33), (Yield: 23% ).\(^1\)H NMR (500 MHz, DMSO-\(d_6\)) \(\delta\) 10.53 and 10.67 (2br. s., 1H), 8.19 (s, 1H), 7.78 and 7.87 (2d, J = 8.7 Hz, 2H, rotamers), 7.29 - 7.39 (m, 2H), 7.17 - 7.28 (m, 3H), 6.98 and 6.94 (2d, J = 8.7 Hz, 2H, rotamers), 4.45 - 4.56 (m, 2H), 4.08 (s, 1H), 3.90 (s, 1H), 3.25 - 3.29 (m, 4H), 2.44 (m, 4H), 2.37 (m, 12.70 Hz, 2H), 2.21 (s, 3H), 1.95 - 2.06 (m, 2H), 1.61 - 1.72 (m, 4H). LCMS 499.01, [M+H]+.
4-(4-methylpiperazin-1-yl)-N-(5-(1-phenylcyclopropanecarbonyl)-1,4,5,6-
tetrahydropyrrolo[3,4-c]pyrazol-3-yl)benzamide, Formic Acid (34), (Yield: 4%). \(^1^H\) NMR (500 MHz, CD\(_3\)OD) \(\delta\) 8.54 (s, 1H), 7.81 and 7.86 (2d, \(J = 8.9\) Hz, 2H, rotamers), 7.21-7.37 (m, 5H), 6.99 and 7.02 (2d, \(J = 8.9\) Hz, 2H, rotamers), 4.41-4.72 (m, 4H), 3.35-3.44 (m, 4H), 2.60-2.63 (m, 4H), 2.36 (s, 3H), 1.46-1.48 (m, 2H), 1.22-1.29 (m, 2H). LCMS 471.01, [M+H]^+. 

(S)-N-(5-(3-methyl-2-phenylbutanoyl)-1,4,5,6-tetrahydropyrrolo[3,4-c]pyrazol-3-yl)-4-(4-
methylpiperazin-1-yl)benzamide (35), (Yield: 43%). \(^1^H\) NMR (500 MHz, CD\(_3\)OD) \(\delta\) 7.85 and 7.86 (2d, \(J = 8.6\) Hz, 2H, rotamers), 7.40-7.45 (m, 2H), 7.31-7.34 (m, 2H), 7.24-7.26 (m, 1H), 6.98 and 7.01 (2d, \(J = 8.6\) Hz, 2H, rotamers), 4.90-5.02 (m, 1H), 4.45-4.73 (m, 3H), 3.42 (d, \(J =

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11 Hz, 1H), 3.36-3.39 (m, 4H), 2.70-2.72 (m, 4H), 2.43 (s, 3H), 1.07 (t, J = 6.5 Hz, 3H), 0.71 (d, J = 6.5 Hz, 3H). LCMS 487.01, [M+H]+. αD - 84.9 (c 1.00, CH3OH).

(R)-N-(5-(3-methyl-2-phenylbutanoyl)-1,4,5,6-tetrahydropyrrolo[3,4-c]pyrazol-3-yl)-4-(4-methylpiperazin-1-yl)benzamide (36), (Yield: 37%). 1H NMR (500 MHz, DMSO-d6) δ 10.60 (br. s., 1H), 7.86 and 7.89 (2d, J = 8.7 Hz, 2H, rotamers), 7.35 - 7.44 (m, 2H), 7.31 (t, J = 7.5 Hz, 2H), 7.20 - 7.26 (m, 1H), 6.95 - 7.01 (m, 2H), 4.85 - 4.97 (m, 1H), 4.37 - 4.58 (m, 3H), 3.27 - 3.30 (m, 4H), 2.41 - 2.48 (m, 4H), 2.29 (m, 1H), 2.22 and 2.23 (2s, 3H, rotamers), 0.99 (d, J = 6.5 Hz, 3H), 0.63 (t, J = 6.5 Hz, 3H). LCMS 487.01, [M+H]+. αD + 87.7 (c 0.85, CH3OH).

(S)-4-(4-methylpiperazin-1-yl)-N-(5-(2-phenylbutanoyl)-1,4,5,6-tetrahydropyrrolo[3,4-c]pyrazol-3-yl)benzamide (37), (Yield: 33%). 1H NMR (500 MHz, DMSO-d6) δ 10.67 (br. s.,
1H), 7.87 (d, J = 8.7 Hz, 2H), 7.29 - 7.36 (m, 4H), 7.20 - 7.26 (m, 1H), 6.97 (d, J = 8.7 Hz, 2H),
4.81 - 4.90 (m, 1H), 4.33 - 4.56 (m, 3H), 3.71 (m, 1H), 3.26-3.28 (m, 4H), 2.41-2.43 (m, 4H),
2.20 and 2.21 (2s, 3H, rotamers), 1.93 - 2.02 (m, 1H), 1.63 (m, 1H), 0.79 - 0.85 (m, 3H). LCMS
473.01, [M+H]^+. α_D - 44.9 (c 0.35, CH3OH).

(R)-4-(4-methylpiperazin-1-yl)-N-(5-(2-phenylpropanoyl)-1,4,5,6-tetrahydropyrrolo[3,4-
c]pyrazol-3-yl)benzamide, formate salt (38), (Yield: 18% ). 1H NMR (500 MHz, DMSO-d6) δ
10.67 (br. s., 1H), 8.19 (s, 1H), 7.83 - 7.90 (m, 2H), 7.30 - 7.35 (m, 4H), 7.22 (m, 1H), 6.95 and
6.98 (2d, J = 8.7 Hz, 2H, rotamers), 4.77 - 4.87 (m, 1H), 4.15 - 4.53 (m, 3H), 3.97 (m, 1H),3.26-
3.28 (m, 4H), 2.42 - 2.43 (m, 4H), 2.21 (s, 3H), 1.33 (d, J = 6.8 Hz, 3H). LCMS 459.01,
[M+H]^+. α_D + 9.7 (c 0.25, CH3OH).
(S)-N-(5-(2-methoxy-3-phenylpropanoyl)-1,4,5,6-tetrahydropyrrolo[3,4-c]pyrazol-3-yl)-4-(4-methylpiperazin-1-yl)benzamide, formate salt (39), (Yield: 23%). $^1$H NMR (400 MHz, CD$_3$OD) δ 8.26 (s, 1H), 7.89 and 7.92 (2d, 8.7 Hz, 2H, rotamers), 7.23 - 7.33 (m, 4H), 7.15 - 7.22 (m, 1H), 7.09 and 7.11 (2d, 8.7 Hz, 2H, rotamers), 4.54 - 4.78 (m, 2H), 4.27 - 4.54 (m, 2H), 4.05 (d, $J = 14.6$ Hz, 1H), 3.56-3.59 (m, 4H), 3.34 (d, $J = 2.9$ Hz, 3H), 3.25-3.29 (m, 4H), 2.99 - 3.10 (m, 2H), 2.84 and 2.85 (2s, 3H, rotamers). LCMS 489.01, [M+H]$^+$. $\alpha_D$ - 5.7 (c 0.26, CH$_3$OH).

(R)-N-(5-(2-methoxy-3-phenylpropanoyl)-1,4,5,6-tetrahydropyrrolo[3,4-c]pyrazol-3-yl)-4-(4-methylpiperazin-1-yl)benzamide, formate salt (40), (Yield: 15%). $^1$H NMR (400 MHz, CD$_3$OD) δ 8.16 (s, 1H), 7.90 and 7.93 (2d, 8.7 Hz, 2H, rotamers), 7.23 - 7.32 (m, 4H), 7.16 -
7.22 (m, 1H), 7.10-7.12 (2d, 9.5 Hz, 2H, rotamers), 4.52 - 4.77 (m, 2H), 4.26 - 4.52 (m, 2H),
4.05 (d, J = 13.1 Hz, 1H), 3.59-3.62 (m, 4H), 3.33-3.36 (m, 4H), 3.34 (d, J = 2.9 Hz, 3H), 2.99 -
3.10 (m, 2H), 2.91 (s, 3H). LCMS 489.01, [M+H]^+. α_D + 4.7 (c 0.14, CH_3OH).

(R)-N-(5-(2-methoxy-4-phenylbutanoyl)-1,4,5,6-tetrahydropyrrolo[3,4-c]pyrazol-3-yl)-4-(4-
methyypiperazin-1-yl)benzamide, formate salt (41), (Yield: 27% ). ^1H NMR (500 MHz,
CD_3OD) δ 8.25 ( s., 1H), 7.87 - 7.97 (m, 2H), 7.16 - 7.32 (m, 4H), 7.05 - 7.15 (m, 3H), 4.43 -
4.82 (m, 4H), 4.43 - 4.58, 3.95 and 4.04 (dd, J = 4.8, 7.8 Hz, 1H), 3.57-3.64 (m, 4H), 3.36 (s,
3H), 2.88 (s, 3H), 2.80 - 2.85 (m, 1H), 2.71 - 2.79 (m, 1H), 1.95 - 2.10 (m, 2H). LCMS 503.01,
[M+H]^+. α_D + 20.7 (c 0.45, CH_3OH).
4-(4-methylpiperazin-1-yl)-N-(5-(1-phenylcyclohexanecarbonyl)-1,4,5,6-tetrahydropyrrolo[3,4-c]pyrazol-3-yl)benzamide, formate salt (42), (Yield: 28%). \( ^1\)H NMR (500 MHz, DMSO-\( d_6 \)) \( \delta \) 10.49 and 10.61 (2br. s., 1H, rotamers), 8.17 (s, 1H), 7.79 and 7.87 (2d, \( J = 8.3 \) Hz, 2H, rotamers), 7.29 - 7.38 (m, 4H), 7.17 - 7.27 (m, 1H), 6.94 and 6.98 (2d, \( J = 8.3 \) Hz, 2H, rotamers), 4.52 (m, 2H), 4.11 (s, 1H), 3.88 (s, 1H), 3.25-3.28 (m, 4H), 2.43-2.45 (m, 4H), 2.34 - 2.42 (m, 2H), 2.22 (s, 3H), 1.57 - 1.71 (m, 7H), 1.30 (m, 1H). LCMS 513.01, [M+H]\(^+\).

(S)-4-(4-methylpiperazin-1-yl)-N-(5-(2-phenylpropanoyl)-1,4,5,6-tetrahydropyrrolo[3,4-c]pyrazol-3-yl)benzamide, formate salt (43), (Yield: 35%). \( ^1\)H NMR (400 MHz, CD\(_3\)OD) \( \delta \) 8.32 (s, 1H), 7.85 - 7.92 (m, 2H), 7.30 - 7.40 (m, 4H), 7.25 (m, 1H), 7.07 (d, \( J = 8.7 \) Hz, 2H),
4.94 (d, \( J = 13.1 \) Hz, 1H), 4.26 - 4.74 (m, 3H), 3.94 - 4.07 (m, 1H), 3.50 - 3.53 (m, 4H), 3.05 - 3.14 (m, 4H), 2.71 and 2.72 (2s, 3H, rotamers), 1.46 (dd, \( J = 4.0, 6.9 \) Hz, 3H). LCMS 459.01, [M+H]\(^+\). \( \alpha_D - 25.9 \) (c 0.12, CH\(_3\)OH).

![Diagram of compound 44](NEU-547)

(S)-N-(5-(2-methoxy-2-phenylacetyl)-1,4,5,6-tetrahydropyrrolo[3,4-c]pyrazol-3-yl)-4-(4-methylpiperazin-1-yl)benzamide (44), (Yield 33%). \(^1\)H NMR (500 MHz, CD\(_3\)OD) \( \delta \) 7.87 - 7.92 (m, 2H), 7.48 - 7.56 (m, 2H), 7.33 - 7.45 (m, 3H), 7.07 and 7.09 (2d, \( J = 8.7 \) Hz, 2H, rotamers), 5.09 (d, \( J = 11.2 \) Hz, 1H), 4.51-4.78 (m, 4H), 3.49 - 3.73 (m, 4H), 3.41 and 3.42 (2s, 3H, rotamers), 3.34 - 3.40 (m, 4H), 2.92 and 2.93 (2s, 3H, rotamers). LCMS 475.01, [M+H]\(^+\). \( \alpha_D - 27.9 \) (c 0.93, CH\(_3\)OH).

![Diagram of compound 45](NEU-577)

N-(5-(2-(6-methoxynaphthalen-2-yl)acetyl)-1,4,5,6-tetrahydropyrrolo[3,4-c]pyrazol-3-yl)-4-(4-methylpiperazin-1-yl)benzamide, formate salt (45). \(^1\)H NMR (500 MHz, DMSO-\( d_6 \)) \( \delta \)
10.67 (br. s., 1H), 8.16 (s, 1H), 7.85 - 7.91 (m, 2H), 7.73 - 7.81 (m, 2H), 7.69 (d, J = 9.2 Hz, 1H), 7.34 - 7.42 (m, 1H), 7.28 (s, 1H), 7.11-7.14 (m, 1H), 6.98 (d, J = 7.8 Hz, 2H), 4.79 (s, 1H), 4.73 (s, 1H), 4.48 (d, J = 14.6 Hz, 2H), 3.83 - 3.88 (m, 5H), 3.26 - 3.31 (m, 4H), 2.43 - 2.47 (m, 4H), 2.23 (s, 3H). LCMS 525.01, [M+H]^+.

N-(5-(3-methyl-2-(naphthalen-2-yl)butanoyl)-1,4,5,6-tetrahydropyrrolo[3,4-c]pyrazol-3-yl)-4-(4-methylpiperazin-1-yl)benzamide (50) , (Yield: 25%). ¹H NMR (500 MHz, DMSO-d6) δ 10.54 and 10.61 (2br. s., 1H, rotamers), 8.53 (dd, J = 8.5, 14.8 Hz, 1H), 8.16 (s, 1H), 7.93 (dd, J = 8.0, 11.9 Hz, 1H), 7.80 - 7.88 (m, 3H), 7.65 - 7.74 (m, 1H), 7.47 - 7.65 (m, 3H), 6.94 - 7.00 (m, 2H), 5.05 (dd, J = 4.3, 12.7 Hz, 1H), 4.53 (t, J = 13.4 Hz, 1H), 4.28 - 4.45 (m, 2H), 3.83 (d, J = 13.1 Hz, 1H), 3.23 - 3.33 (m, 4H), 2.36 - 2.47 (m, 5H), 2.22 and 2.23 (2s, 3H, rotamers), 1.12 (dd, J = 6.6, 10 Hz, 3H), 0.55 (t, J = 6.8 Hz, 3H). LCMS 537.01, [M+H]^+.

N-(5-(3-methyl-2-(naphthalen-2-yl)butanoyl)-1,4,5,6-tetrahydropyrrolo[3,4-c]pyrazol-3-yl)-4-(4-methylpiperazin-1-yl)benzamide (50) (NEU-582)
Other Danusertib analogs exploring the solvent exposed region

(R)-N-(5-(2-methoxy-2-phenylacetyl)-1,4,5,6-tetrahydropyrrolo[3,4-c]pyrazol-3-yl)acrylamide (47), (Yield 46%). \(^1\)H NMR (500 MHz, DMSO-\(d_6\)) \(\delta\) 12.37 (br. s., 1H), 10.67 (br. s., 1H), 7.31 - 7.45 (m, 5H), 6.42 (dd, \(J = 10.2, 16.8\) Hz, 1H), 6.24 (d, \(J = 16.8\) Hz, 1H), 5.67 - 5.78 (m, 1H), 5.03 - 5.11 (m, 1H), 4.80 (m, 1H), 4.35 - 4.59 (m, 3H). LCMS 327.01, [M+H]\(^+\).

\(\alpha_D\) - 177.2 (c 0.40, CH\(_3\)OH).

4-(dimethylamino)-N-(5-(2-(naphthalen-1-yl)acetyl)-1,4,5,6-tetrahydropyrrolo[3,4-c]pyrazol-3-yl)benzamide (48), (Yield: 41%). \(^1\)H NMR (500 MHz, DMSO-\(d_6\)) \(\delta\) 7.97 - 8.04 (m, 1H), 7.92 (m, 1H), 7.88 (d, \(J = 7.8\) Hz, 2H), 7.83 (d, \(J = 7.8\) Hz, 1H), 7.49 - 7.55 (m, 2H), 7.42 - 7.48 (m, 1H), 7.36 - 7.41 (m, 1H), 6.71 (dd, \(J = 2.93, 8.7\) Hz, 2H), 4.83-4.89 (m, 2H), 4.43 - 4.52 (m, 2H), 4.20 (d, \(J = 8.3\) Hz, 2H), 2.98 (s, 6H). LCMS 440.01, [M+H]\(^+\).
N-(5-(2-(naphthalen-1-yl)acetyl)-1,4,5,6-tetrahydropyrrolo[3,4-c]pyrazol-3-yl)acetamide (49), (Yield: 57%). $^1$H NMR (500 MHz, DMSO-$d_6$) $\delta$ 12.21 (br. s., 1H), 10.42 (br. s., 1H), 7.96 - 8.01 (m, 1H), 7.90 - 7.93 (m, 1H), 7.82 (d, $J = 8.3$ Hz, 1H), 7.50 - 7.53 (m, 2H), 7.42 - 7.47 (m, 1H), 7.37 (t, $J = 7.3$ Hz, 1H), 4.76-4.78 (m, 2H), 4.37 - 4.50 (m, 2H), 4.16 (s, 2H), 1.97 (s, 3H). LCMS 335.01, [M+H]$^+$. 

Synthesis of Auk-TAG compounds.
sodium 4-(4-(2-hydroxyethyl)piperazin-1-yl)benzoate (51). Ethyl 4-(4-(2-hydroxyethyl) piperazin-1-yl)benzoate (532 mg, 1.91 mmol) was dissolved in sodium hydroxide (1.1 equiv.) solution (1 M) and MeOH and heated under reflux for 3 h. The solvent was concentrated to give the desired product as a white solid (Yield: 98%). $^1$H NMR (500 MHz, DMSO-$d_6$) δ 7.69 (d, $J = 8.3$ Hz, 2H), 6.76 (d, $J = 8.7$ Hz, 2H), 3.47 - 3.56 (m, 2H), 3.30 (d, $J = 11.7$ Hz, 1H), 3.06 - 3.14 (m, 4H), 2.50 - 2.54 (m, 4H), 2.40 (t, $J = 6.3$ Hz, 2H). LCMS found 251.06, [M+H]$^+$

tert-butyldiphenylsilyl4-(4-((tert-butyldiphenylsilyloxy)ethyl)piperazin-1-yl)benzoate (52). To sodium 4-(4-(2-hydroxyethyl)piperazin-1-yl)benzoate (51) (520 mg, 1.9 mmol) was added imidazole (518 mg, 7.61 mmol), I$_2$ (966 mg, 3.81 mmol) and THF (6 mL). Then tert-butyldiphenylchlorosilane (1.46 ml, 5.71 mmol) was added in one portion. The reaction mixture
was stirred at rt over the weekend. The solvent was then evaporated, the residue dissolved in ethyl acetate and washed with aqueous Na₂S₂O₃ (10%). The organic phase was dried over anhydrous Na₂SO₄ and evaporated. The crude product was confirmed via LCMS found 728.33, [M+H]⁺. The crude mixture was taken to the next step without further characterization.

![Diagram](image)

4-(4-(2-((tert-butyldiphenylsilyl)oxy)ethyl)piperazin-1-yl)benzoic acid (53).

Tert-butyl diphenylsilyl 4-(4-(2-((tert-butyldiphenylsilyl)oxy)ethyl)piperazin-1-yl)benzoate (52) (1.50 g, 2.063 mmol) was dissolved in 1:1 THF/water (10 mL), basified by addition of K₂CO₃ (0.285 g, 2.063 mmol) and heated at 50 °C for 2 h. The reaction was then cooled to room temperature and acidified to pH = 3 with 1 M HCl and extracted with DCM (3 x 100 ml). The organic layers were combined and washed with brine (50 mL) and water (50 mL), dried (MgSO₄), filtered and concentrated at reduced pressure. The crude product was chromatographed 0-10% MeOH in DCM to give the desired product as a light yellowish solid (Yield: 56%). ¹H NMR (500 MHz, CDCl₃) δ 7.95 (d, J = 8.7 Hz, 2H), 7.64 - 7.69 (m, 4H), 7.35 - 7.46 (m, 6H), 6.86 (d, J = 9.2 Hz, 2H), 3.89 (t, J = 5.6 Hz, 2H), 3.34 - 3.40 (m, 4H), 2.79 - 2.86 (m, 4H), 2.74 (t, J = 5.6 Hz, 2H), 1.05 (s, 9H). LCMS found 489.08, [M+H]⁺.
4-(4-(2-((tert-butyldiphenylsilyl)oxy)ethyl)piperazin-1-yl)benzoyl chloride (54). Oxalyl chloride (0.65 ml, 7.45 mmol) was added to a suspension of 4-(4-(2-((tert-butyldiphenylsilyl)oxy)ethyl)piperazin-1-yl)benzoic acid (53) (520 mg, 1.06 mmol) in DCM and DMF (2 drops). The mixture was heated and refluxed for 5h. The solvent was concentrated and the crude was taken to the next step without further characterization.
5-tert-butyl 1-ethyl 3-(4-(4-(2-((tert-butyldiphenylsilyl)oxy)ethyl)piperazin-1-yl)benzamido) pyrrolo [3,4-c]pyrazole-1,5(4H,6H)-dicarboxylate (55). 4-(4-(2-((tert-butyldiphenylsilyl)oxy)ethyl)piperazin-1-yl)benzoyl chloride (54) (537 mg, 1.05 mmol) in THF was added portion wise to a solution of 5-tert-butyl 1-ethyl 3-aminopyrrolo[3,4-c]pyrazole-1,5(4H,6H)-dicarboxylate (46a) (280 mg, 0.94 mmol) in pyridine at 0°C. The reaction mixture was then allowed to warm up to rt and stir for 12 h. After solvent removal, the residue was taken up with EtOAc and the organic layer was washed with sat. Na₂CO₃ and brine and dried over Na₂SO₄. The solvent was evaporated. The crude product was chromatographed using 0-70% EtOAc in hexanes to give the desired product as a tan solid (Yield: 71%). ¹H NMR (500 MHz, CDCl₃) δ 8.53 (s, 1H), 7.74 (d, J = 8.7 Hz, 2H), 7.67 - 7.71 (m, 4H), 7.36 - 7.46 (m, 5H), 6.84 - 6.94 (m, 2H), 4.67 - 4.77 (m, 4H), 4.46 - 4.53 (m, 2H), 3.84 (t, J = 5.8 Hz, 2H), 3.27 - 3.34 (m, 4H), 2.60 - 2.67 (m, 6H), 1.51 (s, 9H), 1.43 (t, J = 7.0 Hz, 3H), 1.05 (s, 9H). LCMS found 768.34, [M+H]⁺.
ethyl 3-(4-(4-(2-((tert-butyldiphenylsilyl)oxy)ethyl)piperazin-1-yl)benzamido)-5,6-dihydro
pyrrolo [3,4-c]pyrazole-1(4H)-carboxylate, hydrochloride salt (56). 4 M HCl (2.9 mL, 11.9
mmol) in dioxane was added dropwise to a stirred solution of 5-tert-butyl 1-ethyl 3-(4-(4-(2-
((tert-butyldiphenylsilyl)oxy)ethyl)piperazin-1-yl)benzamido)pyrrolo[3,4-c]pyrazole-1,5(4H,6H)
dicarboxylate (55) (510 mg, 0.66 mmol) in DCM. The resulting mixture was stirred at room
temperature for 24 h. Then, the reaction mixture was diluted with ether and the solid was filtered
and extensively washed with ether to give the desired product as a white solid. LCMS found
667.28 [M+H]^+. The crude product was taken to the next step without further characterization.
(R)-ethyl 3-(4-(4-(2-((tert-butyldiphenylsilyl)oxy)ethyl)piperazin-1-yl)benzamido)-5-(2-methoxy-2-phenylacetyl)-5,6-dihydropyrrolo[3,4-c]pyrazole-1(4H)-carboxylate (57). To ethyl 3-(4-(4-(2-((tert-butyldiphenylsilyl)oxy)ethyl)piperazin-1-yl)benzamido)-5,6-dihydropyrrolo[3,4-c]pyrazole-1(4H)-carboxylate, hydrochloride salt (56) (468 mg, 0.66 mmol) in DMF (5 mL) was added DIEA (0.581 ml, 3.33 mmol) and slowly (R)-2-methoxy-2-phenylacetyl chloride (246 mg, 1.331 mmol) in DCM (1 mL). The reaction was stirred at room temperature for 5 hours. Then water was added, and the aqueous layer was extracted with DCM (3x), the organics combined, washed with water (1x), brine (1x) and dried under sodium sulfate. The crude was chromatographed using 0-60% EtOAc in hexanes to give the desired product as a light yellow oil (Yield: 56%). ¹H NMR (500 MHz, CDCl₃) δ 8.88 (s, 1H), 7.72 - 7.78 (m, 2H), 7.64 - 7.70 (m, 4H), 7.56 (d, J = 7.3 Hz, 2H), 7.30 - 7.44 (m, 9H), 6.83 - 6.91 (m, 2H), 5.02 (s, 1H), 4.91 - 5.00 (m, 2H), 4.74 - 4.85 (m, 2H), 4.40 - 4.46 (m, 2H), 3.79 - 3.86 (m, 2H), 3.43 and 3.49 (2s, 3H, rotamers), 3.26 - 3.32 (m, 4H), 2.60 - 2.65 (m, 6H), 1.37 - 1.41 (m, 3H), 1.03 (s, 9H). LCMS found 816.28, [M+H]⁺.
(R)-ethyl 3-(4-(4-(2-hydroxyethyl)piperazin-1-yl)benzamido)-5-(2-methoxy-2-phenylacetyl)-5,6-dihydropyrrolo[3,4-c]pyrazole-1(4H)-carboxylate (59). (R)-ethyl 3-(4-(4-(tert-butyldiphenylsilyl)oxyethyl)piperazin-1-yl)benzamido)-5-(2-methoxy-2-phenylacetyl)-5,6-dihydropyrrolo[3,4-c]pyrazole-1(4H)-carboxylate (57) (185 mg, 0.227 mmol) was dissolved in dry THF and TBAF (0.250 ml, 0.250 mmol) was added. The progress of the reaction was monitored by LCMS. After 1 h the reaction mixture was evaporated. The crude residue was chromatographed using 0-10% MeOH in DCM to give the desired product as a white solid (Yield: 79%). $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 9.78 and 10.06 (2br. s., 1H, rotamers), 7.80 and 7.84 (2d, $J = 8.3$ Hz, 2H, rotamers), 7.47 (d, $J = 6.5$ Hz, 1H), 7.40 (d, $J = 6.5$ Hz, 1H), 7.27 - 7.33 (m, 3H), 6.77 (dd, $J = 8.7$, 13.6 Hz, 2H), 4.95 - 5.01 (m, 1H), 4.60 - 4.92 (m, 2H), 4.44 - 4.60 (m, 2H), 4.26 (q, $J = 5.86$ Hz, 2H), 4.16 - 4.22 (m, 2H), 3.33 and 3.40 (2s, 3H, rotamers), 3.23 (m, 4H), 2.65 - 2.70 (m, 2H), 2.52-2.58 (m, 4H), 1.28 - 1.32 (m, 3H). LCMS found 577.13, [M+H]$^+$. Another minor product obtained was (R)-ethyl 3-(4-(4-(2-hydroxyethyl)piperazin-1-yl)benzamido)-5-(2-methoxy-2-phenylacetyl)-5,6-dihydropyrrolo[3,4-c]pyrazole-1(4H)-carboxylate (58) as a white solid (Yield: 5%). $^1$H NMR (500 MHz, CD$_3$OD) $\delta$ 7.84 and 7.86.
(2d, \(J = 4.4\) Hz, 2H, rotamers), 7.46 - 7.56 (m, 2H), 7.34 - 7.43 (m, 3H), 7.01 (d, \(J = 8.7\) Hz, 2H), 5.07 - 5.12 (m, 1H), 4.75 (dd, \(J = 8.7, 13.6\) Hz, 1H), 4.47 - 4.71 (m, 3H), 3.72 - 3.79 (m, 2H), 3.42 and 3.43 (2s, 3H, rotamers), 3.38 - 3.41 (m, 4H), 2.75 - 2.80 (m, 4H), 2.67 (t, \(J = 5.86\) Hz, 2H). LCMS found 505.17, [M+H]\(^{+}\).

(R)-ethyl 3-(4-(4-(2-azidoethyl)piperazin-1-yl)benzamido)-5-(2-methoxy-2-phenylacetyl)-5,6-dihydropyrrolo[3,4-c]pyrazole-1(4H)-carboxylate (60a). To a suspension of (R)-ethyl 3-(4-(4-(2-hydroxyethyl)piperazin-1-yl)benzamido)-5-(2-methoxy-2-phenylacetyl)-5,6-dihydropyrrolo[3,4-c]pyrazole-1(4H)-carboxylate (59) (9.0 mg, 0.016 mmol) in DMF was added DBU (3.53 µl, 0.023 mmol) and lastly diphenyl phosphorazidate (5.05 µl, 0.023 mmol). The reaction mixture was heated to 110 °C and the reaction progress was monitored by LCMS. The starting material was consumed after 2 h. The solvent was concentrated. The crude was chromatographed using 0-5% MeOH in DCM to give the desired product as a white solid (yield: 76%). LCMS found 602.30, [M+H]\(^{+}\). The product was confirmed by LCMS and taken to the next step without further analysis.
(R)-ethyl 3-(4-(4-(2-azidoethyl)piperazin-1-yl)benzamido)-5-(2-methoxy-2-phenylacetyl)-5,6-dihydropyrrolo[3,4-c]pyrazole-1(4H)-carboxylate (60). To (60a) (7.10 mg, 0.012 mmol) was added methanol and TEA (0.230 ml, 1.652 mmol). The reaction mixture was stirred at 55 °C for 48 h. Then, the solvent was concentrated. The crude was purified via Preparative TLC 9:1 DCM:MeOH to give the desired product as a white solid (Yield: 40%). 1H NMR (500 MHz, CD3OD) δ 7.53 and 7.55 (2d, J = 5.9 Hz, 2H, rotamers), 7.34 - 7.49 (m, 5H), 6.97 - 7.02 (m, 2H), 4.94 - 5.04 (m, 1H), 4.59 - 4.80 (m, 2H), 4.08 - 4.48 (m, 2H), 3.73 - 3.77 (m, 2H), 3.36 - 3.39 (m, 4H), 3.33 and 3.40 (2s, 3H, rotamers), 2.76 - 2.82 (m, 4H), 2.69 (q, 2H). LCMS found 530.37, [M+H]+.

(R)-ethyl 5-(2-methoxy-2-phenylacetyl)-3-(4-(4-(2-(prop-2-yn-1-yloxy)ethyl)piperazin-1-yl)benzamido)-5,6-dihydropyrrolo[3,4-c]pyrazole-1(4H)-carboxylate (61a). To a suspension of (R)-ethyl 3-(4-(4-(2-hydroxyethyl)piperazin-1-yl)benzamido)-5-(2-methoxy-2-phenylacetyl)-5,6-dihydropyrrolo[3,4-c]pyrazole-1(4H)-carboxylate (59) (10 mg, 0.017 mmol) in DMF was added DBU (7.84 µl, 0.052 mmol) and lastly 3-bromoprop-1-yn (7.73 µl, 0.069 mmol). The reaction
mixture was heated to 110 °C and the progress of the reaction was monitored by LCMS. The reaction was run for 2h. Then, the solvent was concentrated. The crude was chromatographed using 0-10% MeOH in DCM to give the desired product as a light yellow oil (Yield: 47%). LCMS found 615.30, [M+H]⁺. The product was confirmed by LCMS and taken to the next step without further analysis.

(R)-N-(5-(2-methoxy-2-phenylacetyl)-1,4,5,6-tetrahydropyrrolo[3,4-c]pyrazol-3-yl)-4-(4-(2-(prop-2-yn-1-yloxy)ethyl)piperazin-1-yl)benzamide (61). To (R)-ethyl 5-(2-methoxy-2-phenylacetyl)-3-(4-(4-(2-(prop-2-yn-1-yloxy)ethyl)piperazin-1-yl)benzamido)-5,6-dihydro pyrrolo[3,4-c]pyrazole-1(4H)-carboxylate (61a) (5 mg, 8.13 µmol) was added methanol and TEA (0.098 mL, 0.7 mmol). The reaction was stirred at 50 °C for 60 h. Then, the solvent was concentrated. The crude product was purified via Preparative TLC 9:1 DCM: MeOH to give the desired product as a white solid (Yield: 50%). ¹H NMR (500 MHz, CD₃OD) δ 7.82 - 7.87 (m, 2H), 7.48 - 7.56 (m, 2H), 7.33 - 7.45 (m, 3H), 7.00 - 7.06 (m, 2H), 5.07- 5.11 (m, 1H), 4.77 - 4.95 (m, 2H), 4.55- 4.64 (m, 2H), 3.76 (t, J = 5.8 Hz, 2H), 3.67- 3.69 (m, 1H), 3.54 - 3.59 (m, 1H), 3.38 - 3.47 (m, 4H), 3.42 and 3.44 (2s, 3H, rotamers), 3.01 - 3.04 (m, 1H), 2.78-2.84 (m, 4H), 2.70 (m, 2H). LCMS found 543.37, [M+H]⁺.

Synthesis of AT-9283 and analogs.
(3,4-dinitrophenyl)(morpholino)methanone (63). A solution of 3,4-dinitrobenzoic acid (62) (5.1 g, 24 mmol) in DMF (few drops) and THF (40 mL) was treated with thionyl chloride (2.3 mL, 31.5 mmol), and then was heated and refluxed for 2 hours. The mixture was cooled to 0°C, and TEA (5.02 ml, 36.0 mmol) was added over 20 min. Morpholine (3.66 ml, 42.0 mmol) was added over 15 min, and then the mixture was warmed to ambient temperature and stirred for 24 h. The reaction mixture was then diluted with water (100 mL) and cooled in ice. The solid was filtered off, washed with a further portion of cold water (40 mL) and dried in vacuo to give the product as a yellow solid (Yield: 77%). 1H NMR (500 MHz, DMSO-\(d_6\)) \(\delta\) 8.27 - 8.32 (m, 2H), 8.00 (dd, \(J = 1.4, 8.3\) Hz, 1H), 3.65 (m, 4H), 3.54 (m, 2H), 3.32 (m, 2H). LCMS found 282.14, [M+H]^+.

4-(3,4-dinitrobenzyl)morpholine (64). NaBH\(_4\) (0.848 g, 22.40 mmol) was placed in a nitrogen flushed flask and suspended in THF (30 mL). After the mixture was cooled to approximately 0°C, BF\(_3\).OEt\(_2\) (2.84 mL, 22.40 mmol) was added slowly via syringe. Then, (3,4-dinitrophenyl)(morpholino)methanone (63) (3.0 g, 10.67 mmol) was added as a solid in one portion. The cooling bath was removed and the reaction mixture was stirred at room temperature for 3 hours. The mixture was cooled in an ice-bath, MeOH (20 mL) was added cautiously, and then the mixture was brought to reflux for 1 hour. The mixture was concentrated in vacuo. The residue was partitioned between EtOAc (40 mL) and 1:1 saturated NaHCO\(_3\) solution/ water (40 mL).
organic phase was separated, washed with water (30 mL), and then brine (30 mL), and dried (MgSO₄). The solvent was concentrated. The crude product was chromatographed 0-100 EtOAc in hexane to give the desired product as a yellow solid (Yield: 82%). ¹H NMR (500 MHz, CD₃OD) δ 8.02 - 8.07 (m, 2H), 7.85 (d, J = 8.3 Hz, 1H), 3.70 - 3.72 (m, 4H), 3.68 (s, 2H), 2.48 - 2.51 (m, 4H). LCMS found 268.16, [M+H]⁺.

4-(morpholinomethyl)benzene-1,2-diamine (65). 5% Pd/C (279 mg, 0.131 mmol) and 4-(3,4-dinitrobenzyl)morpholine (64) (700 mg, 2.62 mmol) were suspended in EtOH under nitrogen. The mixture was cooled in ice and then the atmosphere was exchanged for hydrogen. Then the reaction mixture was run at room temperature and the hydrogenation continued at ambient pressure for 12h. The vessel was purged with nitrogen. Then the mixture was filtered through celite, rinsing with EtOH (40 mL) in portions. The solvent was concentrated. The crude product was chromatographed using 0-10% MeOH in DCM to give the desired product as a brown solid (Yield: 87%). ¹H NMR (500 MHz, DMSO-d₆) δ 6.45 (d, J = 1.9 Hz, 1H), 6.39 (d, J = 7.8 Hz, 1H), 6.23 - 6.29 (m, 1H), 4.37 (br. s., 2H), 4.32 (br. s., 2H), 3.51 (m, 4H), 3.16 (s, 2H), 2.26 (m, 4H).
4-((2-(4-nitro-1H-pyrazol-3-yl)-1H-benzo[d]imidazol-5-yl)methyl)morpholine (66).

A mixture of 4-(morpholinomethyl)benzene-1,2-diamine (65) (1.330 g, 6.42 mmol), 4-nitro-1H-pyrazole-3-carboxylic acid (0.907 g, 5.78 mmol), EDC (1.23 g, 6.42 mmol) and HOBT (0.983 g, 6.42 mmol) in dry DMF was stirred at rt for 24 h. Then the mixture was concentrated. To the crude reaction mixture was added acetic acid (15 mL) and the mixture was refluxed for 3 hours. The solvent was removed in vacuo and the residue was purified via chromatography, eluting with 0-10% MeOH in EtOAc to give the product as a yellow solid (Yield: 56%). \(^1\)H NMR (500 MHz, CD\(_3\)OD) \(\delta \) 8.66 (s, 1H), 7.65-7.67 (m, 2H), 7.36 (d, \(J = 7.3 \) Hz, 1H), 3.70 - 3.73 (m, 6H), 2.55 (m, 4H). LCMS found 329.16, [M+H]\(^+\).

3-(5-(morpholinomethyl)-1H-benzo[d]imidazol-2-yl)-1H-pyrazol-4-amine (67).

5% palladium on carbon (42.9 mg, 0.020 mmol) was added to 4-((2-(4-nitro-1H-pyrazol-3-yl)-1H-benzo[d]imidazol-5-yl)methyl)morpholine (66) (189 mg, 0.576 mmol) in DMF (5 mL) under atmosphere of nitrogen. Then, the reaction was run for 5 hours after the atmosphere was first
exchanged for hydrogen. The mixture was filtered through Celite, washing with MeOH (80 mL). The filtrate was concentrated to give the desired product as a brown solid (Yield: 99%). $^1$H NMR (500 MHz, CD$_3$OD) $\delta$ 7.49 - 7.55 (m, 2H), 7.33 (s, 1H), 7.17 (d, $J = 8.3$ Hz, 1H), 3.63 (m, 4H), 3.56 (s, 2H), 2.42 (m, 4H). LCMS found 299.1, [M+H]$^+$.

1-cyclopropyl-3-(3-(5-(morpholinomethyl)-1H-benzo[d]imidazol-2-yl)-1H-pyrazol-4-yl)urea (68, AT-9283). A mixture of 3-(5-(morpholinomethyl)-1H-benzo[d]imidazol-2-yl)-1H-pyrazol-4-amine (67) (172 mg, 0.57 mmol) and CDI (184 mg, 1.13 mmol) in THF was refluxed for 16 h. A mixture was filtered off, and the solvent was concentrated. The crude product was dried under high vacuum. Then the crude product was dissolved in DMF (4 mL), cyclopropylamine (0.488 ml, 6.92 mmol) was added and the reaction was stirred at 100 °C in a sealed vial for 12 h. Then the solvent was concentrated and the product was purified using a C18 column, eluting with 0-35% acetonitrile in water to give the desired product as a light yellow solid (130 mg, 59%). $^1$H NMR (500 MHz, CD$_3$OD) $\delta$ 8.06 (s, 1H), 7.43 - 7.60 (m, 2H), 7.19 (d, $J = 7.8$ Hz, 1H), 3.65-3.67 (m, 4H), 3.58 (s, 2H), 2.64 - 2.71 (m, 1H), 2.44-2.47 (m, 4H), 0.92-0.96 (m, 2H), 0.62-0.65 (m, 2H). LCMS found 382.12, [M+H]$^+$. 

![Chemical Structure](image_url)
(R)-2-methoxy-N-(3-(5-(morpholinomethyl)-1H-benzo[d]imidazol-2-yl)-1H-pyrazol-4-yl)-2-phenylacetamide (69). 3-(5-(morpholinomethyl)-1H-benzo[d]imidazol-2-yl)-1H-pyrazol-4-amine (67) (60.0 mg, 0.2 mmol) in THF (1.5 mL) and DIEA (0.077 ml, 0.442 mmol) was added to (R)-2-methoxy-2-phenylacetyl chloride (74.3 mg, 0.402 mmol) slowly. After the mixture was stirred for 3 hours at rt 0.6 mL of 1N KOH/MeOH solution was added and the mixture stirred for another 16 h. The solvent was concentrated. The crude product was purified via preparative HPLC to give the desired product as a light yellow oil (Yield: 71%). \(^1\)H NMR (500 MHz, DMSO-d\(_6\)) \(\delta\) 13.02 (br. s., 1H), 11.37 (s, 1H), 8.25 (s, 1H), 8.16 (s, 1H), 7.62 (br. s., 1H), 7.45 (d, \(J = 7.3\) Hz, 3H), 7.38 (t, \(J = 7.5\) Hz, 2H), 7.30 - 7.35 (m, 1H), 7.20 (d, \(J = 7.3\) Hz, 1H), 4.94 (s, 1H), 3.64 (s., 2H), 3.57-3.61 (m, 4H), 3.51 (s, 3H), 2.42-2.46 (m, 4H). LCMS found 447.01, [M+H]\(^+\). \(\alpha_D^{21}\) - 168.6 (c 2.30, CH\(_3\)OH).
N-(3-(5-(morpholinomethyl)-1H-benzo[d]imidazol-2-yl)-1H-pyrazol-4-yl)ethane sulfonamide (70). To 3-(5-(morpholinomethyl)-1H-benzo[d]imidazol-2-yl)-1H-pyrazol-4-amine (67) (40.0 mg, 0.13 mmol) in THF (1.5 mL), was added DIEA (0.052 ml, 0.295 mmol) and then ethanesulfonyl chloride (0.025 ml, 0.268 mmol). After the mixture was stirred for 3 hours at rt 0.6 mL of 1N KOH/MeOH solution was added and the mixture stirred for another 16 h. The solvent was concentrated. The crude product was chromatographed using 0-10% MeOH in DCM to give the desired product as a yellow solid (Yield: 12.03%). $^1$H NMR (500 MHz, DMSO-$d_6$) $\delta$ 13.38 (br. s., 1H), 12.99 (br. s, 1H), 7.82 (s, 1H), 7.55-7.62 (m, 1H), 7.41 (br. s., 1H), 7.14-7.20 (m, 1H), 3.54-3.59 (m, 6H), 3.09 - 3.16 (m, 2H), 2.34-2.38 (m, 4H), 1.15 (t, $J = 7.3$ Hz, 3H). LCMS found 391.01, [M+H]$^+$. 

(NEU-663)
APPENDIX 2

Supplemental Synthetic Methods and Characterization for Chapter 3
Experimental procedures and characterization data for Chapter 3 compounds

Tadalafil analogs

General procedure B.

Preparation of Pictet-Spengler products.

The solution with the appropriate tryptophan methyl ester (71A, 1 equiv.) and the desired aldehyde (2.5 equiv.) in DCM was first cooled to 0 °C followed by the drop wise addition of TFA (2.5 equiv.). Then, the reaction mixture was warmed to rt and stirred for 24-48 h. The progress of the reaction was monitored by LCMS. Then, the crude reaction mixture was basified by aqueous NaHCO₃ and extracted with DCM (3x), the combined organics were washed with brine, and dried under sodium sulfate. Generally, the crude products were chromatographed using hexane/EtOAc to give the desired products 71.

![Diagram showing the transformation from 71A to 71a (R¹=H) and 71b (R¹=Br)]
To the solution of 71 (1 equiv.) in DCM was added TEA (5 equiv.), and lastly chloroacetyl chloride (3 equiv.) and the reaction mixture was stirred at rt for 12 h. Then, the solvent was concentrated and the crude products were chromatographed using hexane/EtOAc to give the desired chloroacetyl derivatives 72.

\[
\begin{align*}
71 &\quad R^1 = H \\
71a &\quad R^1 = H \\
71b &\quad R^1 = Br
\end{align*}
\]

TEA (5 equiv.) was added to the solution of methylamine hydrochloride (5 equiv.) in MeOH (3 mL). Then, the solution of 72 (1 equiv.) in MeOH (2 mL) was added to the amine solution. The reaction mixture was heated under reflux for 12-24 h. Then, the solvent was concentrated and the crude residue was partitioned between saturated NH₄Cl and EtOAc. The aqueous layer was extracted with EtOAc (3x), the organics were combined and washed with brine and dried under sodium sulfate. The crude products were chromatographed using hexane/EtOAc or MeOH/DCM to give the piperazinedione analogs 73 or 75. Tadalafil (86) and 87 were synthesized as previously reported in the literature.¹¹⁵
**General procedure C.** Preparation of the tricyclic analogs.

To the solution of ammonium hydroxide (2 mL) was added the solution of 76a-79a (1 equiv.) in dioxane (1 mL). The reaction mixture was stirred at rt for 24 h. Then most of the solvent was evaporated, and the crude residue was partitioned between saturated NH₄Cl and EtOAc. The aqueous was extracted with EtOAc (3x). The combined organics were washed with brine and dried under sodium sulfate. The sodium sulfate was filtered off and the solvent was evaporated. The residue was recrystallized from ethyl acetate to give the desired tricyclic analogs 76-79.
Benzyl(((12aR)-2-methyl-1,4-dioxo-1,2,3,4,6,7,12,12a-octahydropyrazino[1',2':1,6]pyrido[3,4-b]indol-6-yl)methyl)carbamate (73a), (synthesized using General procedure B, Yield: 65%). $^1$H NMR (500 MHz, CDCl$_3$) Diastereomer 1: $\delta$ 8.84 (s, 1H), 7.55 (d, $J = 8.0$ Hz, 1H), 7.38 (d, $J = 8.0$ Hz, 1H), 7.33-7.30 (m, 5H), 7.20 (t, $J = 7.5$ Hz, 1H), 7.15 (t, $J = 7.5$ Hz, 1H), 5.63 (m, 1H), 5.14 (s, 1H), 5.07 (q, $J = 12$ Hz, 2H), 4.15 (m, 1H), 4.10 (s, 2H), 3.94 (m, 1H), 3.63 (dd, $J = 15.5$ Hz, $J = 4$ Hz, 1H), 3.47 (m, 1H), 3.06 (s, 3H), 2.95 (dd, $J = 15.5$ Hz, $J = 11.75$ Hz, 1H). Diastereomer 2: $\delta$ 8.76 (s, 1H), 7.45 (d, $J = 7.5$ Hz, 1H), 7.34-7.28 (m, 5H), 7.19 (t, $J = 7.5$ Hz, 1H), 7.11 (t, $J = 7.5$ Hz, 1H), 5.92 (m, 1H), 5.57 (t, $J = 6.25$ Hz, 1H), 5.14 (d, $J = 12.0$ Hz, 1H), 5.03 (d, $J = 12.5$ Hz, 1H), 4.42 (m, 1H), 4.05 (d, $J = 17$ Hz, 1H), 3.93 (d, $J = 17.5$ Hz, 1H), 3.78 (m, 1H), 3.47 (m, 2H), 2.99 (s, 3H), 2.83 (dd, $J = 16.0$ Hz, $J = 10.0$ Hz, 1H). LCMS found 433.01, [M+H]$^+$. 
(12aR)-2-methyl-6-(pyridin-3-yl)-2,3,12,12a-tetrahydropyrazino[1',2':1,6]pyrido[3,4-b]indole-1,4(6H,7H)-dione (73b), (synthesized using General procedure B, Yield: 54%). Major Diastereomer: $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 8.81 (s br, 1H), 8.70 (d, $J = 1.9$ Hz, 1H), 8.36 (d, $J = 4.4$ Hz, 1H), 7.63 (d, $J = 7.3$ Hz, 1H), 7.46 (d, $J = 7.8$ Hz, 1H), 7.30 (d, $J = 7.3$ Hz, 1H), 7.14 - 7.22 (m, 2H), 7.08 - 7.14 (m, 1H), 6.24 (s, 1H), 4.34 (dd, $J = 3.9$, 11.2 Hz, 1H), 4.11 (d, $J = 17.5$ Hz, 1H), 3.92 (d, $J = 17.6$ Hz, 1H), 3.85 (dd, $J = 4.4$, 16.1 Hz, 1H), 3.22 (dd, $J = 11.7$, 15.6 Hz, 1H), 3.04 (s, 3H). LCMS found 347.01, [M+H]$^+$. 

![Diagram](NEU-256)

(6R,12aR)-6-(benzo[d][1,3]dioxol-5-yl)-2,7-dimethyl-2,3,12,12a-tetrahydropyrazino[1',2':1,6]pyrido[3,4-b]indole-1,4(6H,7H)-dione (74a). To sodium hydride (60 % dispersion in mineral oil, 2.7 mg, 0.067 mmol) in THF (0.5 mL) was added tadalafil (8.7 mg, 0.022 mmol) in dry THF(0.2 mL). The reaction was stirred for 35 min at room temperature before adding iodomethane (4.19 µl, 0.067 mmol), and was then stirred for 3 more hours. To the reaction was then added water and EtOAc. The aqueous layer was extracted with EtOAc (3x). The organics were combined and washed with brine (1x) and finally dried under Na$_2$SO$_4$. The solvent was concentrated. The crude material was chromatographed using 70-100% EtOAc in hexane to provide the desired product as a white solid (Yield: 38%). $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 7.53
(6S,12aR)-6-(benzo[d][1,3]dioxol-5-yl)-2,7-dimethyl-2,3,12,12a-tetrahydropyrazino[1',2':1,6]pyrido[3,4-b]indole-1,4(6H,7H)-dione (74b). To sodium hydride (60 % dispersion in mineral oil, 3.3 mg, 0.083 mmol) in THF (0.5 mL) was added 87 (10.8 mg, 0.028 mmol) in dry THF(0.2 mL). The reaction was stirred for 35 min at room temperature before adding iodomethane (5.20 µl, 0.083 mmol), and was then stirred for 3 more hours. To the reaction was then added water and EtOAc. The aqueous layer was extracted with EtOAc (3x). The organics were combined and washed with brine (1x) and finally dried under Na₂SO₄. The solvent was concentrated. The crude material was chromatographed using 70-100% EtOAc in hexane to provide the desired product as a white solid (Yield: 39%).

$^1$H NMR (500 MHz, CDCl₃) δ 7.53 (d, J = 7.5 Hz, 1H), 7.29 (t, J = 7.7 Hz, 1H), 7.25 (m, 1H), 7.16 (t, J = 7.5 Hz, 1H), 7.02 (s, 1H), 6.80 (s, 1H), 6.71 (d, J = 8 Hz, 1H), 6.65 (d, J = 8 Hz, 1H), 5.95 (s, 2H), 4.32 (dd, J= 12 Hz, J = 4.25 Hz, 1H), 4.15 (d, J = 18.2 Hz, 1H), 4.01 (d, J = 18.5 Hz, 1H), 3.55 (dd, J= 15.5 Hz, J = 4.5 Hz,
1H), 3.42 (s, 3H), 3.00 (s, 3H), 2.95 (dd, J= 15.5 Hz, J = 12.5 Hz, 1H). LCMS found 404.01, [M+H]^+.

(6S,12aR)-6-(benzo[d][1,3]dioxol-5-yl)-10-bromo-2-methyl-2,3,12,12a-tetrahydropyrazino[1',2':1,6] pyrido[3,4-b]indole-1,4(6H,7H)-dione (75), synthesized using General procedure B, Yield 69%). 1H NMR (500 MHz, CDCl₃) δ 8.43 (s, 1H), 7.70 (d, J = 2.0 Hz, 1H), 7.30-7.27 (m, 1H), 7.26 (d, J = 8.5 Hz, 1H), 7.0 (s, 1H), 6.81 (d, J = 2.0 Hz, 1H), 6.75-6.71 (m, 2H), 5.90 (d, J = 6.0 Hz, 2H), 4.37 (dd, J= 11.7 Hz, J = 4.2 Hz, 1H), 4.17 (d, J = 17.5 Hz, 1H), 4.03 (d, J = 17.5 Hz, 1H), 3.53 (dd, J= 15.5 Hz, J = 4.5Hz, 1H), 3.05 (s, 3H), 2.94 (dd, J= 13. 5 Hz, J = 10Hz, 1H). 13C NMR (126 MHz, CDCl₃) δ 165.6, 162.0, 148.6, 135.4, 132.2, 131.6, 128.4, 126.0, 122.8, 121.5, 113.7, 113.1, 109.5, 109.1, 108.8, 101.8, 52.7, 52.1, 51.9, 33.9, 27.8. LCMS found 468.01, [M+H]^+. 

[NEU-259]
(6R,12aR)-6-(benzo[d][1,3]dioxol-5-yl)-10-bromo-2-methyl-2,3,12,12a-tetrahydropyrazino[1',2':1,6]pyrido[3,4-b]indole-1,4(6H,7H)-dione (75a), (synthesized using General procedure B, Yield: 32%). ¹H NMR (500 MHz, CDCl₃) δ 7.84 (s, 1H), 7.72 (d, J = 2.0 Hz, 1H), 7.27-7.24 (m, 1H), 7.13 (d, J = 9.0 Hz, 1H), 6.83 (d, J = 6.5 Hz, 1H), 6.71 (d, J = 2.0 Hz, 1H), 6.70 (d, J = 8.0 Hz, 1H), 6.13 (s, 1H), 5.88 (d, J = 9.0 Hz, 2H), 4.29 (dd, J = 11.5 Hz, 4.5Hz, 1H), 4.12 (d, J = 17.5 Hz, 1H), 3.94 (d, J = 17.5 Hz, 1H), 3.71 (dd, J = 16.5 Hz, J = 4.25 Hz, 1H), 3.18 (dd, J = 16.0 Hz, J = 12.0Hz, 1H), 3.04 (s, 3H). LCMS found 468.01, [M+H]^+.

(6S,12aR)-6-(Benzo[d][1,3]dioxol-5-yl)-2-methyl-10-(1H-pyrazol-5-yl)-2,3,12,12a-tetrahydropyrazino[1',2':1,6]pyrido[3,4-b]indole-1,4(6H,7H)-dione (84). To 75 (24.8 mg,
0.053 mmol) was added 1H-pyrazol-5-ylboronic acid (14.8 mg, 0.132 mmol), and 1,1'-bis(diphenylphosphino) ferrocene-palladium dichloride (7.7 mg, 10.6 µmol). Then 1,4-dioxane was added (1 mL) and lastly aqueous sodium carbonate (2M, 0.117 mmol). The reaction mixture was heated at 90 °C overnight. The reaction was cooled to ambient temperature, and the mixture was diluted with DCM and filtered through celite. The filtrate was concentrated under reduced pressure and the residue was purified by silica gel chromatography (0-5% MeOH in DCM) to give the product as a white solid (Yield: 22%). H NMR (500 MHz, DMSO-d6) δ 11.10 (br, 1H), 7.90 (s, 1H), 7.62-7.50 (m, 3H), 7.33 (d, J = 8.5 Hz, 1H), 6.87 (d, J = 8.0 Hz, 1H), 6.82 (s, 1H), 6.77 (d, J = 2.0 Hz, 1H), 6.63 -6.61 (m, 2H), 6.0 (d, J = 6.5 Hz, 2H), 4.24 (d, J = 18.5 Hz, 1H), 4.08 (dd, J= 11. 5 Hz, J = 4.2Hz, 1H), 4.04 (d, J = 17.5 Hz, 1H), 3.29 (m, 1H), 2.98 (dd, J= 15 Hz, J = 11Hz, 1H), 2.85 (s, 3H). LCMS found 456.01, [M+H]+.

(1R,3R)-1-(Benzo[d][1,3]dioxol-5-yl)-2,3,4,9-tetrahydro-1H-pyrido[3,4-b]indole-3-carboxamide, (76). To the solution of ammonium hydroxide (2.06 mL) was added a solution of 76a (0.041 g, 0.118 mmol) in dioxane (1 mL). The mixture was stirred at room temperature overnight. Then, the reaction was concentrated and the residue was partitioned between sat. NH4Cl and EtOAc. The aqueous layer was extracted with EtOAc (3x). The organics were combined and washed with brine (1x), dried over Na2SO4. After filtration and evaporation, the
residue was recrystallized from EtOAc to give the product as a pale yellow solid (Yield: 10%).\textsuperscript{1}H NMR (500 MHz, CDCl\textsubscript{3}) $\delta$ 7.55 (d, J = 8 Hz, 1H), 7.46 (s br, 1H), 7.24 (d, J = 7.5 Hz, 1H), 7.17-7.11 (m, 2H), 6.85-6.81 (m, 3 H), 6.78 (s, 1H), 5.97 (s, 2H), 5.41 (s br, 1H), 5.17 (s, 1H), 3.79 (dd, J= 11.5 Hz, J = 4.5 Hz, 1H), 3.37 (m, 1H), 2.90 (m, 1H). LCMS found 336.16, [M+H]\textsuperscript{+}.

(1S,3R)-1-(benzo[d][1,3]dioxol-5-yl)-2,3,4,9-tetrahydro-1H-pyrido[3,4-b]indole-3-carboxamide (77). To a solution of ammonium hydroxide (2.01 mL) was added a solution of 77\textsuperscript{a} (0.033 g, 0.094 mmol) in dioxane (1 mL). The mixture was stirred at room temperature overnight. Then, the reaction was concentrated and the residue was partitioned between sat. NH\textsubscript{4}Cl and EtOAc. The aqueous layer was extracted with EtOAc (3x), and the organics were combined and washed with brine (1x), and dried over Na\textsubscript{2}SO\textsubscript{4}. After filtration and evaporation, the residue was recrystallized from EtOAc to give the desired product as a pale yellow solid (Yield: 27%). \textsuperscript{1}H NMR (500 MHz, CDCl\textsubscript{3}) $\delta$ 7.68 (s, 1H), 7.58 (d, J = 7.5 Hz, 1H), 7.29 (d, J = 8 Hz, 1H), 7.19 (t, J = 6.5 Hz, 1H), 7.14 (t, J = 6.5 Hz, 1H), 6.82 (s br, 1H), 6.76 (s, 1H), 6.73 (d, J = 7.5 Hz, 1H), 6.64 (d, J = 7.5 Hz, 1H), 5.95 (s, 2H), 5.36 (s br, 1H), 5.22 (s,1H), 3.69 (m, 1H), 3.30 (dd, J= 15.5 Hz, J = 5 Hz, 1H), 2.95 (dd, J= 15.5 Hz, J = 8.5 Hz, 1H). LCMS found 336.01, [M+H]\textsuperscript{+}. 

\textsuperscript{1}H
1-methyl-2,3,4,9-tetrahydro-1H-pyrido[3,4-b]indole-3-carboxamide (78), (synthesized using General procedure C, 31%). $^1$H NMR (500 MHz, DMSO-$d_6$) $\delta$ 10.77 (s, 1H), 7.38 (br, 1H), 7.36 (d, $J = 8$ Hz, 1H), 7.27 (d, $J = 8$ Hz, 1H), 7.14 (s br, 1H), 7.01 (t, $J = 7.5$ Hz, 1H), 6.94 (t, $J = 7.5$ Hz, 1H), 4.12 (m, 1H), 3.46 (m, 1H), 2.93 (m, 1H), 2.56 (m, 1H), 1.41 (d, $J = 7.0$ Hz, 3H). LCMS found 230.01, [M+H]$^+$. 

(3R)-1-(pyridin-3-yl)-2,3,4,9-tetrahydro-1H-pyrido[3,4-b]indole-3-carboxamide (79), (synthesized using General procedure C, Yield: 20%). $^1$H NMR (500 MHz, DMSO-$d_6$) Diastereomer 1: $\delta$ 10.40 (s, 1H), 8.60 (d, $J = 1.5$ Hz, 1H), 8.53 (dd, $J = 4.5$ Hz, $J = 1.7$ Hz, 1H), 7.70 (m, 1H), 7.42 (m, 1H), 7.36 (br, 2H), 7.19 (d, $J = 8.0$ Hz, 1H), 7.00-6.95 (m, 3H), 5.24 (d, $J = 7.5$ Hz, 1H), 3.58-3.55 (m, 1H), 3.04 (m, 1H), 2.75 (m, 1H). Diastereomer 2: $\delta$ 10.75 (s, 1H), 8.47 (d, $J = 2.0$ Hz, 1H), 8.45 (dd, $J = 4.5$ Hz, $J = 1.5$ Hz, 1H), 7.54 (m, 1H), 7.37 (dd, $J = 7.5$ Hz,
J = 4.5 Hz, 1H), 7.36 (m, 1H), 7.24 (d, J = 8.0 Hz, 1H), 7.11 (s br, 1H), 7.06-7.00 (m, 3H), 5.29 (d, J = 4.0 Hz, 1H), 3.16 (m, 1H), 2.9 (dd, J = 15.5 Hz, J = 5 Hz, 1H), 2.72 (m, 1H). LCMS found 293.01, [M+H]^+.

(12aR)-6-(aminomethyl)-2-methyl-2,3,12,12a-tetrahydropyrazino[1',2':1,6]pyrido[3,4-b]indole-1,4(6H,7H)-dione (80). To 73a (154 mg, 0.356 mmol) in EtOH (4 mL) was added 5% palladium on carbon (38 mg, 0.018 mmol) under nitrogen. Then the atmosphere was exchanged for hydrogen and the mixture was stirred for 3 hours at ambient pressure under hydrogen. After the reaction completed as determined by TLC the mixture was filtered through Celite, rinsing with EtOH (20 mL) in portions. The solvent was concentrated to provide 80 as a light yellow solid (Yield: 93%).^1^H NMR (500 MHz, CDCl₃) Diastereomer 1: δ 8.70 (s, 1H), 7.61 (d, J = 8.0 Hz, 1H), 7.40 (d, J = 8.0 Hz, 1H), 7.25-7.17 (m, 2H), 5.24 (m, 1H), 4.14 (d, J = 17.5 Hz, 1H), 4.00 (d, J = 17.5 Hz, 1H), 3.71 (q, 1H), 3.67 (dd, J = 15.7 Hz, J = 4.7 Hz, 1H), 3.51 (dd, J = 15.0 Hz, J = 4.0 Hz, 1H), 3.09 (s, 3H), 2.93-2.89 (m, 2H), 2.08 (br, 2H). Diastereomer 2: δ 9.08 (s, 1H), 7.50 (d, J = 8.0 Hz, 1H), 7.37 (d, J = 7.5 Hz, 1H), 7.17-7.12 (m, 2H), 5.65 (m, 1H), 4.38 (dd, J = 11.5 Hz, J = 3.5 Hz, 1H), 4.12-4.05 (m, 2H), 3.30 (dd, J = 12.5 Hz, J = 3.5 Hz, 1H), 3.27 (dd, J = 11.5 Hz, J = 5.5 Hz, 1H), 3.07 (s, 3H), 2.93-2.89 (m, 2H), 2.08 (br, 2H). LCMS found 299.18, [M+H]^+. 
N-(((12aR)-2-methyl-1,4-dioxo-1,2,3,4,6,7,12,12a-octahydropyrazino[1',2':1,6]pyrido[3,4-b]indol-6-yl)methyl)acetamide (81). To 80 (12 mg, 0.040 mmol) was added dry THF (1 mL), then DIEA (15.6 mg, 0.121 mmol), and lastly acetic anhydride (4.5 mg, 0.044 mmol). The reaction was stirred overnight at room temperature. The solvent was concentrated and the crude residue was chromatographed using 9:1 DCM:MeOH to provide 81 (Yield: 53%). \(^1\)H NMR (500 MHz, CDCl\(_3\)) Diastereomer 1: \(\delta\) 9.14 (s, 1H), 7.45 (d, \(J = 7.5\) Hz, 1H), 7.35 (d, \(J = 8.5\) Hz, 1H), 7.14-7.08 (m, 2H), 6.61 (m, 1H), 4.42 (dd, \(J = 12.0\) Hz, \(J = 4.0\) Hz, 1H), 4.12 (d, \(J = 18.0\) Hz, 1H), 4.05 (m, 1H), 4.03 (d, \(J = 17.5\) Hz, 1H), 3.94 (dd, \(J = 6.5\) Hz, \(J = 3\) Hz, 1H), 3.49 (dd, \(J = 16.0\) Hz, \(J = 4.5\) Hz, 1H), 3.43-3.39 (m, 1H), 3.03 (s, 3H), 2.85 (dd, \(J = 15.5\) Hz, \(J = 10\) Hz, 1H), 2.00 (s, 3H). Diastereomer 2: \(\delta\) 9.61 (s, 1H), 7.52 (d, \(J = 7.5\) Hz, 1H), 7.40 (d, \(J = 7.5\) Hz, 1H), 7.20-7.16 (m, 2H), 6.81 (m, 1H), 5.04 (s, 1H), 4.16 (dd, \(J = 11.5\) Hz, \(J = 4.5\) Hz, 1H), 4.11 (m, 1H), 3.97 (dd, \(J = 6.5\) Hz, \(J = 3.5\) Hz, 1H), 3.91 (dd, \(J = 7.5\) Hz, \(J = 3\) Hz, 1H), 3.62 (dd, \(J = 16.0\) Hz, \(J = 4.5\) Hz, 1H), 3.49-3.45 (m, 1H), 3.05 (s, 3H), 2.93 (dd, \(J = 15.5\) Hz, \(J = 10\) Hz, 1H), 1.99 (s, 3H). LCMS found 341.01, [M+H]^+.
3-Methyl-N-(((12aR)-2-methyl-1,4-dioxo-1,2,3,4,6,7,12,12a-octahydropyrazino[1',2':1,6]pyrido[3,4-b]indol-6-yl)methyl)-1H-pyrazole-4-carboxamide (82). To 80 (14.2 mg, 0.048 mmol) was added 3-methyl-1H-pyrazole-4-carboxylic acid (6.0 mg, 0.048 mmol), EDC (21.9 mg, 0.114 mmol), HOBT (8.7 mg, 0.057 mmol), DCM (1 mL) and lastly TEA (0.033 ml, 0.238 mmol). The reaction was placed in the shaker and stirred for 3 days. The solvent was concentrated. The crude was chromatographed using 0-10% MeOH in DCM to provide 82 (Yield: 22%). ¹H NMR (500 MHz, CD₃OD) δ 7.78 (br, 1H), 7.45 (d, J = 8.0 Hz, 1H), 7.34 (d, J = 8.0 Hz, 1H), 7.12 (t, J = 7.5 Hz, 1H), 7.03 (t, J = 7.5 Hz, 1H), 6.03 (dd, J= 10.0 Hz, J = 2.7 Hz, 1H), 4.62 (dd, J= 12.0 Hz, J = 4.5 Hz, 1H), 4.19 (d, J = 18.0 Hz, 1H), 3.98 (d, J = 18.0 Hz, 1H), 3.93 (dd, J= 14.5 Hz, J = 4.0 Hz, 1H), 3.65 (m, 1H), 3.37 (d, J = 4.5 Hz, 1H), 3.02 (s, 3H), 2.93 (dd, J= 15.0 Hz, J = 12.0 Hz, 1H), 2.48 (br, 3H). LCMS found 407.01, [M+H]⁺.
(12aR)-2-Methyl-6-(((pyridin-3-ylmethyl)amino)methyl)-2,3,12,12a-tetrahydropyrazino
[1',2':1,6] pyrido[3,4-b]indole-1,4(6H,7H)-dione (83) To nicotinaldehyde (4.7 mg, 0.044
mmol) was added sodium triacetoxyborohydride (28.1 mg, 0.133 mmol), 80 (13.2 mg, 0.044
mmol) and lastly THF (1 mL) and DMF (0.2 mL). The reaction was stirred for 48 hours. The
solvent was concentrated and the residue was purified by preparative TLC (9:1 DCM:MeOH) to
give 83 (Yield:16%). \(^1\)H NMR (500 MHz, CD\(_3\)OD) \(\delta\) 8.51 (s, 1H), 8.42 (d, J = 5.0 Hz, 1H),
7.83 (m, 1H), 7.43 (d, J = 7.5 Hz, 1H), 7.39 (dd, J = 7.5 Hz, J = 4.5 Hz, 1H), 7.31 (d, J = 8.0 Hz, 1H),
7.10 (t, J = 7.5 Hz, 1H), 7.02 (t, J = 7.5 Hz, 1H), 5.96 (t, J = 6.5 Hz, 1H), 4.59 (dd, J = 12.0 Hz, J
= 4.5 Hz, 1H), 4.24 (d, J = 17.5 Hz, 1H), 4.07 (d, J = 17.5 Hz, 1H), 3.98 (d, J = 13.7 Hz, 1H),
3.82 (d, J = 13.7 Hz, 1H), 3.49 (q, 1H), 3.16 (d, J = 6.0 Hz, 2H), 3.03 (s, 3H), 2.89 (dd, J = 15.5
Hz, J = 13.0 Hz, 1H). LCMS found 390.01, [M+H]^+.\)
1-(1-(benzo[d][1,3]dioxol-5-yl)-3,4-dihydro-1H-pyrido[3,4-b]indol-2(9H)-yl)ethanone (85).

To a mixture of $85a^{116}$ (54 mg, 0.185 mmol), and TEA (0.028 mL, 0.2 mmol) in THF (3 mL) was slowly added acetyl chloride (0.014 mL, 0.2 mmol). The reaction mixture was stirred overnight at rt. Then the solvent was concentrated and the crude was chromatographed 0-10% MeOH in DCM to give the desired product as a tan solid. (Yield: 87%). $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 7.76 (s, 1H), 7.52 (d, J = 7.5 Hz, 1H), 7.30 (d, J = 8.0 Hz, 1H), 7.19 (t, J = 7.5 Hz, 1H), 7.13 (t, J = 7.5 Hz, 1H), 6.93 (s, 1H), 6.87 (s, 1H), 6.77 (d, J = 7.5 Hz, 1H), 6.70 (d, J = 8.5 Hz, 1H), 5.92 (s, 2H), 3.90 (dd, J= 14 Hz, J = 4.5 Hz, 1H), 3.46 (m, 1H), 2.94-2.86 (m, 2H), 2.19 (s, 3H). LCMS found 335.01, [M+H]$^+$. 

2,6-dimethyl-2,3,12,12a-tetrahydropyrazino[1',2':1,6]pyrido[3,4-b]indole-1,4(6H,7H)-dione (88), (synthesized using General procedure B, Yield: 64%). $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 8.15 (s, 1H), 7.58 (d, J = 7.5 Hz, 1H), 7.37 (d, J = 8.0 Hz, 1H), 7.20 (t, J = 7.5 Hz, 1H), 7.15 (t, J = 7.5 Hz, 1H), 5.42 (q, 1H), 4.13 (d, J = 14.5 Hz, 1H), 4.10 (m, 1H), 3.99 (d, J = 16.5 Hz, 1H), 2.19 (s, 3H). LCMS found 335.01, [M+H]$^+$. 

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3.69 (dd, J= 15.5 Hz, J = 4.5 Hz, 1H), 3.07 (s, 3H), 2.98 (dd, J= 16.5 Hz, J = 10 Hz, 1H), 1.53 (d, J = 7.0 Hz, 3H). LCMS found 284.01, [M+H]^+.

(3R)-methyl1-((((benzyloxy)carbonyl)amino)methyl)-2,3,4,9-tetrahydro-1H-pyrido[3,4-b]indole-3-carboxylate (89) (synthesized using General procedure B, Preparation of Pictet-Spengler products, Yield 52%). ^1H NMR (500 MHz, CDCl₃) Diastereomer 1: \( \delta \) 8.23 (s br, 1H), 7.48 (d, J = 8.0 Hz, 1H), 7.35-7.16 (m, 7 H), 7.11 (t, J = 7.0 Hz, 1H), 5.32 (br,1H), 5.05-4.99 (m, 2H), 4.38 ( br, 1H), 3.83 (m, 1H), 3.82 (s, 3H), 3.74 (m, 1H), 3.63-3.58 (m, 1H), 3.16- 3.12 (m, 1H), 2.87-2.81 (m, 1H), 2.25 (br, 1H). Diastereomer 2: \( \delta \) 8.08 (s br, 1H), 7.36 (d, J = 8.5 Hz, 1H), 7.35-7.16 (m, 7 H), 7.11 (m, 1H), 5.42 (br, 1H), 5.16-5.08 (m, 2H), 4.37 (br, 1H), 3.91 (m, 1H), 3.79 (m, 1H), 3.74 (s, 3H), 3.42-3.37 (m, 1H), 3.11-3.08 (m, 1H), 2.97-2.92 (m, 1H), 2.25 (br, 1H). LCMS found 394.24, [M+H]^+.
Synthesis of piclamilast analogs.

\[ \text{methyl 3-[(2-(1,3-dioxoisoindolin-2-yl)ethoxy)-4-methoxybenzoate (91)} \]

To methyl 3-hydroxy-4-methoxybenzoate (111 mg, 0.609 mmol) was added triphenylphosphine (192 mg, 0.731 mmol), and then toluene under nitrogen. Then, 2-(2-hydroxyethyl)isoindoline-1,3-dione (151 mg, 0.792 mmol) and DEAD (0.305 mL) were finally added. The reaction mixture was allowed to run for 12 h under nitrogen atmosphere. The solvent was concentrated. The residue was dissolved in DCM. Then NaOH (0.5 M) was added and the aqueous layer was extracted 2x with DCM. The organics were dried under sodium sulfate. The crude product was chromatographed using 0-35% EtOAc in hexane to give the desired product as a white solid (Yield: 47%).

\[ \text{1H NMR (500 MHz, CDCl}_3\text{)} \delta 7.87-7.85 (m, 2H), 7.73-7.71 (m, 2H), 7.67 (dd, J= 8.7 Hz, J = 2.0 Hz, 1H), 7.57 (d, J = 2.5 Hz, 1H), 6.84 (d, J = 8.7 Hz, 1H), 4.32 (t, J = 6.2 Hz, 2H), 4.32 (t, J = 6.0 Hz, 2H), 3.87 (s, 3H), 3.80 (s, 3H). \]
3-(2-(1,3-dioxoisoindolin-2-yl)ethoxy)-4-methoxybenzoic acid (92). To methyl 3-(2-(1,3-dioxoisoindolin-2-yl)ethoxy)-4-methoxybenzoate (225 mg, 0.633 mmol) was added a 1 M solution of NaOH (5 mL) and the reaction mixture was stirred at 90 °C for 1 hour. The residue was dissolved in water and acidified with 1 M HCl to pH 3. The resulting precipitate was filtered off, washed with water and dried in vacuo to give the title compound as a white solid (Yield: 95%). ¹H NMR (400 MHz, DMSO- d₆) δ 8.53 (m, 1H), 7.76 (d, J = 8.8 Hz, 1H), 7.59-7.48 (m, 4H), 7.42 (d, J = 7.2 Hz, 1H), 7.06 (d, J = 8.8 Hz, 1H), 4.10 (t, J = 6.8 Hz, 2H), 3.82 (s, 3H), 3.56 (q, 2H). LCMS found 342.01 [M+H]⁺.

3-(2-aminoethoxy)-N-(3,5-dichloropyridin-4-yl)-4-methoxybenzamide (93). To 3-(2-(1,3-dioxoisoindolin-2-yl)ethoxy)-4-methoxybenzoic acid (884 mg, 2.59 mmol) was added toluene (7
mL) and DMF (a few drops), and then slowly thionyl chloride (0.378 mL, 5.18 mmol). The reaction mixture was stirred at 110 °C. After 5 hours the reaction was complete as determined by IR, and the reaction mixture was cooled to room temperature. The solvent was concentrated to give 3-(2-(1,3-dioxoisindolin-2-yl)ethoxy)-4-methoxybenzoyl chloride. In a different round bottom flask a solution of 3,5-dichloropyridin-4-amine (845 mg, 5.18 mmol) in dry DMF (5.5 mL) was added dropwise to a stirred suspension of sodium hydride (60 % dispersion in mineral oil, 276 mg, 5.18 mmol) in DMF(4 mL), maintaining the temperature below 20 °C. After stirring for 30 minutes a solution of 3-(2-(1,3-dioxoisindolin-2-yl)ethoxy)-4-methoxybenzoyl chloride (932 mg, 2.59 mmol) in DMF (20 mL) was added dropwise keeping the temperature below 10 °C. Then the reaction mixture was heated to 50 °C for 30 minutes. After that the solution was treated with 1 M aq. HCl (50 mL). The mixture was diluted with DCM (100 mL), then the organic phase separated, washed with water (5x), sat. NaHCO₃ (1x, 50 mL) and again with water (1x, 50mL). The organic phase was dried under Na₂SO₄ and concentrated. The crude product was confirmed by LCMS and used to the next step without further characterization. To N-(3,5-dichloropyridin-4-yl)-3-(2-(1,3-dioxoisindolin-2-yl)ethoxy)-4-methoxybenzamide (530 mg, 1.090 mmol) was added methanol (7 mL). Then hydrazine (0.267 mL, 5.45 mmol) was added and the reaction was allowed to stir at room temperature overnight. The solvent was concentrated. The crude residue was dissolved in EtOAc and extracted with 1M HCl (3x). Then, the aqueous layers were combined and 1 M NaOH was added. The aqueous layer was extracted with EtOAc (3x) and the organics were dried under sodium sulfate. The sodium sulfate was filtered off, the solvent concentrated to give the title compound as a white solid (Yield: 44%). ¹H NMR (500 MHz, DMSO- d₆) δ 8.71 (s, 2H), 7.67 (dd, J= 8.7 Hz, J = 2.0 Hz, 1H), 7.58 (d, J =
2.0 Hz, 1H), 7.11 (d, J = 8.7 Hz, 1H), 3.98 (t, J = 6.0 Hz, 2H), 3.84 (s, 3H), 2.90 (t, J = 6.0 Hz, 2H). LCMS found 356.01 [M+H]+.

4-(2-(5-(3,5-dichloropyridin-4-ylcarbamoyl)-2-methoxyphenoxy)ethylamino)-4-oxobutanoic acid (94). To 3-(2-aminoethoxy)-N-(3,5-dichloropyridin-4-yl)-4-methoxybenzamide (25.0 mg, 0.070 mmol) in DCM was added DIEA (0.037 ml, 0.21 mmol) , succinic anhydride (10.5 mg, 0.105 mmol) and the reaction was stirred at 80 °C for 12 hours. Then, the solvent was concentrated. The crude product was purified via preparative HPLC to give the title compound as a white solid (Yield: 25%). ^1H NMR (500 MHz, CD$_3$OD) δ 8.63 (s, 2H), 7.70 (dd, J= 9.0 Hz, J = 2.5 Hz, 1H), 7.63 (d, J = 2.0 Hz, 1H), 7.12 (d, J = 9.0 Hz, 1H), 4.14 (t, J = 5.0 Hz, 2H), 3.94 (s, 3H), 3.61 (q, 2H), 2.55-2.48 (m, 4H). LCMS found 456.01 [M+H]+.
3-(2-acetamidoethoxy)-N-(3,5-dichloropyridin-4-yl)-4-methoxybenzamide (95). To 3-(2-aminoethoxy)-N-(3,5-dichloropyridin-4-yl)-4-methoxybenzamide (23.7 mg, 0.067 mmol) in DCM was added DIEA (0.035 ml, 0.200 mmol) and acetic anhydride (0.013 ml, 0.133 mmol) and the reaction was allowed to run at 80 °C for 2 hours. The solvent was concentrated. The crude was purified using preparative HPLC, and then was further purified via chromatography, eluting with 0-10% MeOH in DCM to give the title compound (Yield: 23%). $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 8.56 (s, 2H), 8.03 (s, 1H), 7.70 (s, 1H), 7.64 (d, J = 8.5 Hz, 1H), 6.99 (d, J = 8.5 Hz, 1H), 6.00 (br, 1H), 4.20 (t, J = 5.5 Hz, 2H), 3.96 (s, 3H), 3.70 (q, 2H), 2.01 (s, 3H). LCMS found 398.01 [M+H]$^+$. 
N-(3,5-dichloropyridin-4-yl)-4-methoxy-3-(2-(methylsulfonamido)ethoxy)benzamide (96). To 3-(2-aminoethoxy)-N-(3,5-dichloropyridin-4-yl)-4-methoxybenzamide (21.0 mg, 0.06 mmol) in DMF was added methanesulfonyl chloride (9.2 µl, 0.118 mmol) and DIEA (0.031 mL, 0.177 mmol). The reaction mixture was run at 80 °C for 2 hours in the shaker. The solvent was concentrated. The crude was purified via preparative HPLC and then was further purified via chromatography, eluting with 0-10% MeOH in DCM to give the title compound as a white solid (Yield: 10%). \(^{1}H\) NMR (500 MHz, CDCl\(_3\)) \(\delta\) 8.56 (s, 2H), 7.71 (s, 1H), 7.61 (dd, \(J=\ 8.5\ \text{Hz, } J = 2.5\ \text{Hz, } 1H\)), 7.57 (d, \(J = 2.5\ \text{Hz, } 1H\)), 6.98 (d, \(J = 8.5\ \text{Hz, } 1H\)), 4.95 (m, 1H), 4.23 (t, \(J = 5.0\ \text{Hz, } 2H\)), 3.95 (s, 3H), 3.58 (q, 2H), 3.04 (s, 3H). LCMS found 434.01 [M+H]\. 
N-(3,5-dichloropyridin-4-yl)-4-methoxy-3-(2-(2-methylbenzamido)ethoxy)benzamide (97).

To 3-(2-aminoethoxy)-N-(3,5-dichloropyridin-4-yl)-4-methoxybenzamide (25.3 mg, 0.071 mmol) was added 2-methylbenzoic acid (10.6 mg, 0.078 mmol), EDC (32.7 mg, 0.170 mmol), HOBT (13.0 mg, 0.085 mmol), DMF (1.5 mL) and DIEA (0.062 ml, 0.35 mmol). The reaction mixture was placed in the shaker and was run at room temperature for 5 hours. Then, the solvent was concentrated. The crude was purified via preparative HPLC to give the title compound (Yield: 27%). $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 8.52 (s, 2H), 8.02 (s, 1H), 7.77 (d, $J = 2.0$ Hz, 1H), 7.62 (dd, $J = 8.7$ Hz, $J = 2.0$ Hz, 1H), 7.35 (d, $J = 7.5$ Hz, 1H), 7.31 (m, 1H), 7.20-7.16 (m, 2H), 6.97 (d, $J = 8.7$ Hz, 1H), 6.41 (m, 1H), 4.30 (t, $J = 5.5$ Hz, 2H), 3.89 (s, 3H), 3.86 (q, 2H), 2.40 (s, 3H). LCMS found 474.01 [M+H]$^+$. 

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N-(3,5-dichloropyridin-4-yl)-4-methoxy-3-(2-(4-methylphenylsulfonamido)ethoxy)benzamide (98). To 3-(2-aminoethoxy)-N-(3,5-dichloropyridin-4-yl)-4-methoxybenzamide (25.3 mg, 0.071 mmol) in DMF was added 4-methylbenzene-1-sulfonyl chloride (20.3 mg, 0.107 mmol) and DIEA (0.037 ml, 0.21 mmol). The reaction mixture was placed in the shaker and was run at room temperature for 5 hours. The solvent was concentrated. The crude was purified via preparative HPLC to give the desired product (Yield: 30%). \(^1\)H NMR (500 MHz, CDCl\(_3\)) \(\delta\) 8.54 (s, 2H), 8.02 (s, 1H), 7.73 (d, \(J = 7.0\) Hz, 2H), 7.62 (dd, \(J = 8.7\) Hz, \(J = 2.0\) Hz, 1H), 7.50 (d, \(J = 2.5\) Hz, 1H), 7.27 (d, \(J = 7.0\) Hz, 2H), 6.95 (d, \(J = 8.7\) Hz, 1H), 5.35 (m, 1H), 4.10 (t, \(J = 5.5\) Hz, 2H), 3.93 (s, 3H), 3.36 (q, 2H), 2.39 (s, 3H). LCMS found 510.01 [M+H].
3-(2-(4-(1H-tetrazol-5-yl)benzamido)ethoxy)-N-(3,5-dichloropyridin-4-yl)-4-methoxy benzamide (99). To 3-(2-aminoethoxy)-N-(3,5-dichloropyridin-4-yl)-4-methoxybenzamide (39.0 mg, 0.109 mmol) was added EDC (50.4 mg, 0.263 mmol), HOBT (20.1 mg, 0.131 mmol) and 4-(1H-tetrazol-5-yl)benzoic acid (37.5 mg, 0.197 mmol). Then DMF was added (3 mL) and lastly DIEA (0.096 ml, 0.547 mmol). The reaction mixture was stirred at room temperature overnight. The solvent was concentrated. The product was purified via preparative HPLC to give the title compound as a white solid (Yield: 12%). $^1$H NMR (400 MHz, $d_4$-CD$_3$OD) $\delta$ 8.62 (s, 2H), 8.14 (d, $J$ = 8.8 Hz, 2H), 7.96 (d, $J$ = 8.8 Hz, 2H), 7.69 (dd, $J$ = 8.0 Hz, $J$ = 2.0 Hz, 1H), 7.67 (d, $J$ = 2.0 Hz, 1H), 7.13 (d, $J$ = 8.0 Hz, 1H), 4.30 (t, $J$ = 5.4 Hz, 2H), 3.94 (s, 3H), 3.85 (t, $J$ = 5.4 Hz, 2H). LCMS found 528.01 [M+H]$^+$. 

![Chemical Structure Image]
N-(3,5-dichloropyridin-4-yl)-3-(2-(3-(2,6-diethylphenyl)ureido)ethoxy)-4-methoxy benzamide (100). To 3-(2-aminoethoxy)-N-(3,5-dichloropyridin-4-yl)-4-methoxybenzamide (25.3 mg, 0.071 mmol) in DMF was added 1,3-diethyl-2-isocyanatobenzene (0.018 ml, 0.107 mmol) and DIEA (0.037 ml, 0.21 mmol). The reaction mixture was placed in the shaker and was run at room temperature overnight. Then the solvent was concentrated. The crude was purified via preparative HPLC to give the title compound as a white solid (Yield: 6%). $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 8.55 (s, 2H), 8.46 (br, 1H), 7.88 (s, 1H), 7.66 (dd, J = 8.2 Hz, J = 2.0 Hz, 1H), 7.24 (m, 1H), 7.13 (m, 2H), 6.95 (d, J = 8.2 Hz, 1H), 5.70 (br, 1H), 4.70 (br, 1H), 4.20 (t, J = 6.0 Hz, 2H), 3.85 (s, 3H), 3.60 (q, 2H), 2.59 (m, 4H), 1.14 (t, J = 7.7 Hz, 6H). LCMS found 531.01 [M+H]$^+$. 

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GSK256066

Synthesis of GSK256066.

![Chemical structure](image)

**ethyl 6-iodo-8-methyl-4-oxo-1,4-dihydroquinoline-3-carboxylate (118).** To 4-iodo-2-methylaniline (5.35 g, 22.96 mmol) was added diethyl 2-(ethoxymethylene)malonate (5.10 mL, 25.3 mmol) and the mixture was heated at 100 °C for 1 hour. The heat was removed and the white solid that formed was collected, washed with cyclohexane (70 mL) and ethanol 30 mL (2x), and dried in vacuo at 40 °C overnight to give the title compound as a white solid (Yield: 98%). \(^1\)H NMR (500 MHz, DMSO-\(d_6\)) \(\delta\) 10.83 (d, \(J = 13.6\) Hz, 1H), 8.42 (d, \(J = 13.1\) Hz, 1H), 7.65 (s, 1H), 7.56 - 7.61 (m, 1H), 7.25 (d, \(J = 8.3\) Hz, 1H), 4.20 (q, \(J = 7.3\) Hz, 2H), 4.11 (q, \(J = 7.3\) Hz, 2H), 2.25 (s, 3H), 1.24 (td, \(J = 6.9, 10.5\) Hz, 6H). LCMS found 404.16 [M+H]^+.

![Chemical structure](image)

**ethyl 6-iodo-8-methyl-4-oxo-1,4-dihydroquinoline-3-carboxylate (119).** To diethyl 2-((4-iodo-2-methylphenylamino)methylene)malonate (9.0 g, 22.3 mmol) was added diphenyl ether (35.5 mL, 223 mmol). The reaction was run for 45 min at 250 °C. The mixture was cooled and isohexane was added (30 mL). The solid formed (light yellow solid) was collected by filtration.
and washed further with isohexane (30 mL). The solid was dried under high vacuum to give the desired product as a light yellow solid (Yield: 100%). 1H NMR (400 MHz, DMSO-\(d_6\)) \(\delta\) 11.74 (br. s., 1H), 8.37 (s, 1H), 8.28 (s, 1H), 7.89 (s, 1H), 4.21 (q, \(J = 7.3\) Hz, 2H), 1.26 (t, \(J = 7.3\) Hz, 3H). LCMS found 357.95 [M+H]+.

4-chloro-6-iodo-8-methylquinoline-3-carboxamide (110). NaOH (1.96 g, 49.1 mmol) was dissolved in water (40 mL) and ethanol (20 mL). The resultant solution was added to 119 (7.97 g, 22.32 mmol) and the mixture was heated and refluxed for 1 hour with stirring. Then concentrated HCl was added until a white precipitate formed. The reaction was stirred overnight at room temperature. After stirring overnight the precipitate was filtered, washed with water and dried in vacuo to give 6-iodo-8-methyl-4-oxo-1,4-dihydroquinoline-3-carboxylic acid as a white solid confirmed by LCMS, LCMS found 329.89 [M+H]+. To 6-iodo-8-methyl-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (7.34 g, 22.30 mmol) was added thionyl chloride (24.42 ml, 335 mmol), then 3 drops DMF were added. The mixture was refluxed for 2 hours. The excess thionyl chloride was evaporated in vacuo and the residue was azeotroped with toluene (5 mL, 1x). The crude product 4-chloro-6-iodo-8-methylquinoline-3-carboxyl chloride was used in the next step without further characterization. To stirred ammonium hydroxide (34.7 ml, 892 mmol) was added portion wise 4-chloro-6-iodo-8-methylquinoline-3-carbonyl chloride (8.16 g, 22.3
mmol) and the mixture was stirred at room temperature overnight. The solid formed was filtered, washed with water and dried under vacuum at 60 °C to give \textbf{110} as a white solid (Yield: 80%).  

$^1$H NMR (400 MHz, DMSO-$d_6$) $\delta$ 8.89 (s, 1H), 8.46 (s, 1H), 8.20 (br. s., 1H), 8.10 (s, 1H), 7.99 (br. s., 1H), 2.70 (s, 3H). LCMS found 346.87 [M+H]$^+$. 

![Image](image1.png)

\textbf{6-iodo-4-(3-methoxyphenylamino)-8-methylquinoline-3-carboxamide (120)}. Compound \textbf{110} (174 mg, 0.499 mmol) was dissolved in acetonitrile and 3-methoxyaniline (0.059 ml, 0.52 mmol) was added. The mixture was heated and refluxed overnight. The precipitate formed was filtered, washed with acetonitrile and the solid (light yellow solid) obtained was dried under high vacuum to give the title compound (Yield: 90%).  

$^1$H NMR (400 MHz, DMSO-$d_6$) $\delta$ 8.78 (s, 1H), 8.39 (br. s., 1H), 8.31 (br. s., 1H), 8.10 (br. s., 1H), 7.74 (br. s., 1H), 7.28 (t, $J=8.0$ Hz, 1H), 6.73 - 6.86 (m, 3H), 3.72 (s, 3H), 2.65 (s, 3H). LCMS found 434.97 [M+H]$^+$. 

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methyl 3-(3-carbamoyl-4-(3-methoxyphenylamino)-8-methylquinolin-6-ythio)benzoate (121). To 120 (20.0 mg, 0.046 mmol) was added Pd2(dba)3 (8.4 mg, 9.23 µmol), then 2,2'-oxybis(2,1-phenylene)bis(diphenylphosphine) (9.9 mg, 0.018 mmol). The methyl 3-mercaptobenzoate (11.6 mg, 0.069 mmol) was dissolved in toluene (1.5 mL) and then added to the solids. Lastly, KOtBu (0.092 ml, 0.092 mmol) was added. The mixture was placed in the M.W. and heated for 40 minutes at 170 °C. The crude product was filtered through celite after adding methanol and further washed with methanol. The solvent was concentrated. The crude product was chromatographed using 50-100% EtOAC in hexane to give the desired product 121 as an orange solid (Yield: 75%). ¹H NMR (400 MHz, CD3OD) δ 8.91 (s, 1H), 7.91 (d, J = 7.3 Hz, 1H), 7.85 (s, 1H), 7.50 (s, 1H), 7.42 - 7.46 (m, 2H), 7.33 - 7.40 (m, 1H), 6.99 (t, J = 8.0 Hz, 1H), 6.49 - 6.53 (m, 1H), 6.30 - 6.36 (m, 2H), 3.91 (s, 3H), 3.66 (s, 3H), 2.69 (s, 3H). LCMS found 474.01, [M+H]+.
methyl 3-(3-carbamoyl-4-(3-methoxyphenylamino)-8-methylquinolin-6-ylsulfonyl)benzoate (122). To 121 (103 mg, 0.218 mmol) was added oxone (401 mg, 0.653 mmol) in DMF (5 mL). The reaction was stirred at room temperature for 5 hours. Then, the reaction mixture was poured into water (40 mL) and extracted with DCM (5x), and the combined organic layers were dried (Na$_2$SO$_4$) and concentrated. The crude product was chromatographed using 0-10 % MeOH in DCM to give the desired product as an orange solid (Yield: 85%). $^1$H NMR (500 MHz, CDCl$_3$)  $\delta$ 10.86 (s, 1H), 8.93 (s, 1H), 8.44 (s, 1H), 8.23 - 8.26 (m, 1H), 8.21 (d, $J = 7.8$ Hz, 1H), 7.91 (s, 1H), 7.77 (d, $J = 7.8$ Hz, 1H), 7.54 (t, $J = 7.8$ Hz, 1H), 7.15 (t, $J = 8.0$ Hz, 1H), 6.75 (dd, $J = 1.95$, 8.3 Hz, 1H), 6.57 (d, $J = 7.8$ Hz, 1H), 6.54 (s, 1H), 5.90-6.10 (br. s.,2H), 3.97 (s, 3H), 3.65 (s, 3H), 2.75 (s, 3H). LCMS found 506.01, [M+H]$^+$. 

![Diagram of molecules](image-url)
3-(3-carbamoyl-4-(3-methoxyphenylamino)-8-methylquinolin-6-ylsulfonyl)benzoic acid (123). A solution of 122 (60.0 mg, 0.119 mmol) in ethanol (2mL) was treated with NaOH (0.890 mL, 1.780 mmol) and the resulting solution was stirred at 45 °C overnight. The solvent was evaporated. The residue was dissolved in water and acidified with 1 M HCl to pH 4. The resulting precipitate was filtered, washed with water and dried in vacuo. The crude product was purified via preparative HPLC to give the desired product as a yellow solid (Yield: 31%). \(^1\)H NMR (400 MHz, CD\(_3\)OD) \(\delta\) 9.01 (s, 1H), 8.38 (s, 1H), 8.34 (s, 1H), 8.24 (d, \(J = 7.3\) Hz, 1H), 7.99 (s, 1H), 7.86 (d, \(J = 8.0\) Hz, 1H), 7.65 (t, \(J = 7.7\) Hz, 1H), 7.18 (t, \(J = 8.0\) Hz, 1H), 6.74 - 6.79 (m, 1H), 6.53 - 6.62 (m, 2H), 3.62 (s, 3H), 2.75 (s, 3H). LCMS found 492.01, [M+H]\(^+\).
6-(3-(dimethylcarbamoyl)phenylsulfonyl)-4-(3-methoxyphenylamino)-8-methylquinoline-3-carboxamide (GSK256066). To 123 (50.0 mg, 0.102 mmol) in DMF was added HATU (42.5 mg, 0.112 mmol). After 5 minutes dimethylamine (0.049 ml, 0.098 mmol) and DIEA (0.037 ml, 0.214 mmol) were added. The resulting solution was stirred at room temperature overnight. The solvent was concentrated. The crude product was chromatographed using 0-10% MeOH in DCM to give the desired product as a light yellow solid (Yield: 21%). $^1$H NMR (400 MHz, DMSO-$d_6$) $\delta$ 10.95 (br. s., 1H), 9.04 (s, 1H), 8.40 (s, 1H), 8.31 (br. s., 1H), 8.04 (br. s., 1H), 7.63 - 7.81 (m, 4H), 7.15 (t, $J = 8.0$ Hz, 1H), 6.68 - 6.76 (m, 2H), 6.55 (d, $J = 8.0$ Hz, 1H), 3.66 (s, 3H), 3.00 (s, 3H), 2.84 (s, 3H), 2.68 (s, 3H). LCMS found 519.13, [M+H]$^+$. 
4-(3-methoxyphenylamino)-8-methyl-6-(3-(methylcarbamoyl)phenylsulfonyl)quinoline-3-carboxamide (124). To 123 (8.6 mg, 0.017 mmol) in DMF was added HATU (7.3 mg, 0.019 mmol). After 5 minutes methanamine, HCl (1.14 mg, 0.017 mmol) and DIEA (6.4 µl, 0.037 mmol) were added. The resulting solution was stirred at room temperature for 6 hours. The solvent was concentrated. The crude product was purified via preparative HPLC to give the desired product as a yellow solid (Yield: 37%). $^1$H NMR (400 MHz, DMSO-$d_6$) $\delta$ 10.77 (s, 1H), 9.08 (s, 1H), 8.71 (d, $J = 4.4$ Hz, 1H), 8.32 - 8.35 (m, 1H), 8.29 (br. s., 1H), 8.26 (s, 1H), 8.08 (d, $J = 8.0$ Hz, 1H), 7.99 (s, 1H), 7.76 (d, $J = 7.3$ Hz, 2H), 7.68 (t, $J = 7.7$ Hz, 1H), 7.12 (t, $J = 8.0$ Hz, 1H), 6.69 (dd, $J = 1.8$, 8.4 Hz, 1H), 6.60 (s, 1H), 6.52 (d, $J = 7.3$ Hz, 1H), 3.62 (s, 3H), 2.81 (d, $J = 4.4$ Hz, 3H), 2.69 (s, 3H). LCMS found 505.01, [M+H]$^+$. 
6-(3-carbamoylphenylsulfonyl)-4-(3-methoxyphenylamino)-8-methylquinoline-3-carboxamide (125). To the solution of ammonium hydroxide (0.761 ml, 19.55 mmol) was added the solution of 122 (18.0 mg, 0.036 mmol) in dioxane. The mixture was stirred at room temperature overnight. Then the solvent was concentrated, and the residue was partitioned between sat. NH₄Cl and ethyl acetate. The aqueous layer was extracted with EtOAc (3x) and the combined organics were washed with brine and dried over Na₂SO₄. After filtration and evaporation, the crude product was chromatographed using 0-10% MeOH to give the desired product as a yellow solid (Yield: 18%). ¹H NMR (400 MHz, CD₃OD) δ 9.02 (s, 1H), 8.36 (s, 1H), 8.31 (s, 1H), 8.10 (s, 1H), 7.99 (s, 1H), 7.81 (d, J = 7.3 Hz, 1H), 7.65 (t, J = 8.0 Hz, 1H), 7.17 (t, J = 8.4 Hz, 1H), 6.75 - 6.80 (m, 1H), 6.55 - 6.60 (m, 2H), 3.64 (s, 3H), 2.75 (s, 3H). LCMS found 491.01, [M+H]⁺.
4-((3-methoxyphenyl)amino)-8-methyl-6-(phenylthio)quinoline-3-carboxamide (126). To compound 120 (40.0 mg, 0.092 mmol) was added Pd$_2$(dba)$_3$ (16.9 mg, 0.018 mmol) and 2,2'-oxybis(2,1-phenylene)bis(diphenylphosphine) (19.9 mg, 0.037 mmol). Then the benzenethiol (0.014 ml, 0.138 mmol) was dissolved in toluene and added to the solids. Lastly, KOtBu (0.115 ml, 0.185 mmol) was added. The mixture was run in the M.W. for 40 minutes at 170 °C. To the crude product was added methanol and the solution was filtered through celite. Then, the solvent was concentrated. The crude product was chromatographed 0-100% EtOAc in hexane to give the desired product as a yellow solid. (Yield: 53%). $^1$H NMR (400 MHz, CD$_3$OD) δ 8.89 (s, 1H), 7.44 (s, 2H), 7.18 - 7.29 (m, 5H), 7.08 (t, $J = 8.0$ Hz, 1H), 6.58 - 6.64 (m, 1H), 6.34 - 6.40 (m, 2H), 3.71 (s, 3H), 2.67 (s, 3H). LCMS found 416.01, [M+H]$^+$. 
4-((3-methoxyphenyl)amino)-8-methyl-6-(phenylsulfonyl)quinoline-3-carboxamide (127).

To compound 126 (13.4 mg, 0.032 mmol) was added oxone (59.5 mg, 0.097 mmol) and DMF (3 mL). The reaction was stirred for 4 h at rt. Then, the reaction mixture was poured into water (20 mL) and extracted with DCM (5x), the combined organics were washed with brine (1x) and dried under sodium sulfate. The crude product was chromatographed using 50-70% EtOAc in hexane to give the desired product as a light yellow solid. (Yield: 62%). $^{1}$H NMR (400 MHz, CD$_3$OD) $\delta$ 9.03 (s, 1H), 8.38 (s, 1H), 7.93 (s, 1H), 7.72 (d, $J = 7.3$ Hz, 2H), 7.64 (t, $J = 7.3$ Hz, 1H), 7.55 (t, $J = 7.7$ Hz, 2H), 7.24 (t, $J = 8.0$ Hz, 1H), 6.82 (dd, $J = 1.8$, 8.4 Hz, 1H), 6.63 (d, $J = 7.3$ Hz, 1H), 6.57 (s, 1H), 3.64 (s, 3H), 2.74 (s, 3H). LCMS found 448.01, [M+H]$^+$.

4-((3-methoxyphenyl)amino)-8-methyl-6-(methylsulfonyl)quinoline-3-carboxamide (129).

4-chloro-8-methyl-6-(methylsulfonyl)-1,4-dihydroquinoline-3-carboxamide (128)$^{110}$ (88 mg, 0.293
mmol) was dissolved in acetonitrile (2 mL) and 3-methoxyaniline (0.035 ml, 0.307 mmol) was added. The mixture was heated in the M.W. at 130 °C for 20 minutes. The precipitate formed was filtered, then washed with acetonitrile (5 mL) and the solid obtained was dried under high vacuum to give 129 as a light yellow solid (Yield: 59%). \(^1\)H NMR (500 MHz, CD\(_3\)OD) δ 8.91 (s, 1H), 8.41 (br. s., 1H), 8.21 (s, 1H), 7.36 - 7.44 (m, 1H), 7.00 (d, \(J = 8.3 \) Hz, 1H), 6.87 - 6.95 (m, 2H), 3.80 (s, 3H), 3.02 (s, 3H), 2.79 (s, 3H). LCMS found 386.01, [M+H]^+.

4-((3,5-dichloropyridin-4-yl)amino)-8-methyl-6-(methylsulfonyl)quinoline-3-carboxamide (130). To NaH (60 % dispersion in mineral oil, 10.6 mg, 0.266 mmol) in DMF (1 mL) was added 3,5-dichloropyridin-4-amine (43.4 mg, 0.266 mmol) in DMF (1 mL) and the reaction mixture was stirred at rt for 1 h. Then 128 (40.0 mg, 0.133 mmol) was added portion wise and the reaction was run at 50 °C for 2 h. To the reaction mixture was added EtOAc (15 mL), and the organic layer was washed with water (3x), brine (1x), and the combined organics were dried under sodium sulfate. The solvent was concentrated to give the desired product as a yellow solid (Yield: 21%). \(^1\)H NMR (500 MHz, CD\(_3\)OD) δ 8.64 (s., 1H), 8.40 ( s., 2H), 7.99 ( s., 1H), 7.94 ( s., 1H), 2.94 ( s., 3H), 2.62 (s, 3H). LCMS found 425.01, [M+H]^+.
methyl 3-(3-carbamoyl-4-(3-methoxyphenylamino)-8-methylquinolin-6-ylamino)benzoate (131). To **120** (40.0 mg, 0.092 mmol) was added 2,2'-oxybis(2,1-phenylene)bis(diphenylphosphine) (19.9 mg, 0.037 mmol), then Pd (dppf) Cl₂·DCM (13.5 mg, 0.018 mmol) and methyl 3-aminobenzoate (27.9 mg, 0.185 mmol). Lastly, KOtBu (0.185 ml, 0.185 mmol) was added. The reaction mixture was run in the M.W. for 20 minutes at 160 °C. After the reaction mixture cooled to ambient temperature the crude was filtered through Celite washing with MeOH (3x). The solvent was concentrated under reduced pressure. Then, the crude product was purified via preparative HPLC to give **131** as a yellow solid (Yield: 12%). 

\[ \text{H NMR (400 MHz, CD}_3\text{OD)} \delta 8.76 (s, 1H), 7.55 (s, 1H), 7.46 (d, } J = 8.0 \text{ Hz, } 1H), 7.30 (d, } J = 2.9 \text{ Hz, } 1H), 7.26 (s, 1H), 7.13 (t, } J = 8.0 \text{ Hz, } 1H), 7.08 (t, } J = 8.0 \text{ Hz, } 1H), 6.95 (dd, } J = 1.5, 8.0 \text{ Hz, } 1H), 6.62 (dd, } J = 1.8, 8.4 \text{ Hz, } 1H), 6.50 (t, } J = 2.2 \text{ Hz, } 1H), 6.43 - 6.47 (m, 1H), 3.88 (s, 3H), 3.70 (s, 3H), 2.70 (s, 3H). \]

LCMS found 457.01, [M+H]⁺.
**General procedure D.** Synthesis of 106 GSK intermediates.

![Chemical structures](image)

To aniline derivatives 102 (1 equiv.) was added diethyl 2-(ethoxymethylene)malonate (1.1 equiv) and the mixture was heated at 100 °C for 1 hour. Then, the heat was removed and the white solid that formed was collected, washed with cyclohexane (3x) and ethanol (2x), and dried in vacuo at 40 °C overnight to give 103 as a white solid (Yields: 80-98%). To 103 (1 equiv.) was added diphenyl ether (10 equiv.). The reaction was heated and refluxed at 250 °C for 1 hour. Then, the mixture was cooled and isohexane was added. The solid formed was collected by filtration and washed further with isohexane. The solid was dried under high vacuum to give the product 104 as a light yellow solid that was used without further purification (Yields: 81-100%).

After that to 104 was added NaOH (2.1 equiv) in water/ethanol (2:1). The reaction mixture was heated and refluxed for 1 hour with stirring. Then concentrated HCl was added until a white precipitate formed. The reaction was stirred overnight at room temperature. After stirring...
overnight the precipitate (acid product) was filtered, washed with water and dried in vacuo. To the precipitate (1 equiv.) was added thionyl chloride (15 equiv.), then 3 drops DMF were added. The mixture was refluxed for 2 hours. The excess thionyl chloride was evaporated in vacuo and the residue was azeotroped with toluene (1x). The crude dichlorinated product was added portion wise to stirred ammonium hydroxide (40 equiv.) and the mixture was stirred at room temperature overnight. The solid formed was filtered, washed with water and dried under vacuum at 60 °C to give 105 (Yields: 61-92%). Compound 105 (1 equiv) was dissolved in acetonitrile and the desired amine (1.1 equiv) was added. The mixture was heated and refluxed overnight. The precipitate formed was filtered, washed with acetonitrile and the solid obtained was dried under high vacuum to give 106 analogs.

**General procedure E.** Synthesis of Suzuki products.

To iodo-substituted template 106 (1 equiv.) was added the desired boronic acid (1.5 equiv.), [1,1′-bis(diphenylphosphino)ferrocene]dichloropalladium (II) complex with dichloromethane (0.1 equiv.), dioxane and sodium carbonate (6 equiv.). The reaction mixture was run in the M.W. at 145 °C for 20 minutes. After the reaction mixture cooled to ambient temperature the crude was filtered through celite washing with MeOH/DCM (1:9). The filtrate was concentrated under reduced pressure. Unless otherwise noted the crude products were chromatographed or purified via preparative HPLC to give the desired products 108.
methyl 3-(3-carbamoyl-4-((3-methoxyphenyl)amino)-8-methylquinolin-6-yl)benzoate (132)
(synthesized from 120 using the **General procedure E**, yellow solid, Yield: 19%). $^1$H NMR
(400 MHz, CDCl$_3$) $\delta$ 10.92 (br. s., 1H), 9.03 (s, 1H), 7.96 (s, 2H), 7.81 (d, $J$ = 9.5 Hz, 2H), 7.36 -
7.43 (m, 2H), 7.21 - 7.25 (m, 1H), 6.78 (d, $J$ = 9.5 Hz, 1H), 6.63 - 6.70 (m, 2H), 3.95 (s, 3H),
3.75 (s, 3H), 2.84 (s, 3H). LCMS found 442.01, [M+H]$^+$.

4-((3-methoxyphenyl)amino)-8-methyl-6-(1-methyl-1H-indazol-6-yl)quinoline-3-
carboxamide (133), (synthesized from 120 using the **General procedure E**, yellow solid, Yield:
56%). $^1$H NMR (400 MHz, DMSO-$d_6$) $\delta$ 10.59 (s, 1H), 9.00 (s, 1H), 8.27 (br. s., 1H), 8.02 (s,
2H), 7.94 (br. s., 1H), 7.68 - 7.76 (m, 2H), 7.51 (s, 1H), 7.16 - 7.26 (m, 2H), 6.66 - 6.74 (m, 2H),
6.57 (d, J = 7.3 Hz, 1H), 4.02 (s, 3H), 3.68 (s, 3H), 2.77 (s, 3H). LCMS found 438.01, [M+H]+.

4-((3-methoxyphenyl)amino)-8-methyl-6-(pyrimidin-5-yl)quinoline-3-carboxamide (134),
synthesized from 120 using the General procedure E, yellow solid, Yield: 40%. 1H NMR
(400 MHz, CD3OD) δ 9.09 (s, 1H), 8.96 (s, 1H), 8.79 (s, 2H), 8.00 (s, 1H), 7.93 (s, 1H), 7.25 (t,
J = 8.0 Hz, 1H), 6.76 (dd, J = 2.2, 8.0 Hz, 1H), 6.63 - 6.70 (m, 2H), 3.75 (s, 3H), 2.81 (s, 3H).
LCMS found 386.01, [M+H]+.

4-((3-methoxyphenyl)amino)-8-methyl-6-(3-morpholinophenyl)quinoline-3-carboxamide (135),
synthesized from 120 using the General procedure E, yellow solid, Yield: 49%. 1H NMR
(400 MHz, CDCl3) δ 10.59 (s, 1H), 8.92 (s, 1H), 7.86 (s, 1H), 7.78 (s, 1H), 7.24 (m, 1H),
7.14 - 7.22 (m, 1H), 6.94 (d, J = 7.3 Hz, 1H), 6.86 (dd, J = 2.2, 8.0 Hz, 1H), 6.68 (s, 1H), 6.60 - 6.66 (m, 3H), 3.85 - 3.92 (m, 4H), 3.72 (s, 3H), 3.04 - 3.11 (m, 4H), 2.83 (s, 3H). LCMS found 469.01, [M+H]+.

![Chemical Structure](image)

6-(3-(cyclopentylcarbamoyl)phenyl)-4-((3-methoxyphenyl)amino)-8-methylquinoline-3-carboxamide (136), (synthesized from 120 using the General procedure E, yellow solid, Yield: 20%). $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 10.61 (s, 1H), 8.92 (s, 1H), 7.79 (d, J = 6.6 Hz, 2H), 7.69 (s, 1H), 7.65 (d, J = 8.0 Hz, 1H), 7.37 (t, J = 7.7 Hz, 1H), 7.29 (d, J = 8.0 Hz, 1H), 7.17 - 7.24 (m, 1H), 6.71 (dd, J = 2.2, 8.0 Hz, 1H), 6.59 - 6.66 (m, 2H), 5.98 (d, J = 7.3 Hz, 1H), 4.37 - 4.49 (m, 1H), 3.73 (s, 3H), 2.82 (s, 3H), 2.06 - 2.19 (m, 2H), 1.71 - 1.82 (m, 2H), 1.64 - 1.71 (m, 2H), 1.51 (qd, J = 6.3, 12.5 Hz, 2H). LCMS found 495.01, [M+H]+.
6-(3-aminophenyl)-4-((3-methoxyphenyl)amino)-8-methylquinoline-3-carboxamide (137),
(synthesized from 120 using the General procedure E, reddish solid, Yield: 7%). $^1$H NMR (500 MHz, CD$_3$OD) $\delta$ 8.89 (s, 1H), 7.88 - 7.94 (m, 1H), 7.82 (s, 1H), 7.22 (t, $J$ = 8.0 Hz, 1H), 7.07 (t, $J$ = 7.8 Hz, 1H), 6.78 (s, 1H), 6.70 - 6.74 (m, 1H), 6.67 (d, $J$ = 7.8 Hz, 2H), 6.60 - 6.64 (m, 2H), 3.72 (s, 3H), 2.78 (s, 3H). LCMS found 399.01, [M+H]$^+$.

4-((3-methoxyphenyl)amino)-8-methyl-6-(1H-pyrazol-4-yl)quinoline-3-carboxamide (138),
(synthesized from 120 using the General procedure E, yellow solid, Yield: 10%). $^1$H NMR (400 MHz, DMSO-$d_6$) $\delta$ 10.29 (s, 1H), 8.90 (s, 1H), 8.25 (s, 1H), 8.16 (br. s., 1H), 7.85 (s, 1H), 7.79 (s, 1H), 7.64 (br. s., 1H), 7.16 (t, $J$ = 8.0 Hz, 1H), 6.59 - 6.65 (m, 2H), 6.53 (d, $J$ = 8.0 Hz, 1H), 3.67 (s, 3H), 2.69 (s, 3H). LCMS found 374.01, [M+H]$^+$.
4-((3-methoxyphenyl)amino)-6-(6-methoxypyridin-3-yl)-8-methylquinoline-3-carboxamide (139), (synthesized from 120 using the General procedure E, yellow solid, Yield: 5%). \(^1\)H NMR (500 MHz, CD\(_3\)OD) \(\delta\) 8.93 (s, 1H), 8.12 (d, \(J = 2.4\) Hz, 1H), 7.88 - 7.90 (m, 1H), 7.84 (s, 1H), 7.71 (dd, \(J = 2.4, 8.8\) Hz, 1H), 7.23 (t, \(J = 8.0\) Hz, 1H), 6.81 (d, \(J = 8.8\) Hz, 1H), 6.72 - 6.75 (m, 1H), 6.61 - 6.66 (m, 2H), 3.93 (s, 3H), 3.73 (s, 3H), 2.80 (s, 3H). LCMS found 415.01, [M+H]\(^+\).

4'-(3-methoxyphenylamino)-8'-methyl-3,6'-biquinoline-3'-carboxamide, Formic Acid (140), (synthesized from 120 using the General procedure E, yellow solid, Yield: 18%). \(^1\)H NMR (500 MHz, DMSO-\(d_6\)) \(\delta\) 10.54 (br. s., 1H), 9.01 (s, 1H), 8.94 (d, \(J = 1.9\) Hz, 1H), 8.41 (d, \(J = 1.9\) Hz, 1H), 8.24 (br. s., 1H), 8.13 (s, 1H), 8.09 (s, 1H), 8.03 (d, \(J = 8.3\) Hz, 1H), 7.96 (d, \(J = 7.8\) Hz, 1H), 7.74 - 7.80 (m, 1H), 7.70 (br. s., 1H), 7.63 - 7.68 (m, 1H), 7.23 (t, \(J = 8.0\) Hz, 1H), 6.70 -
6.75 (m, 1H), 6.69 (s, 1H), 6.61 (d, J = 7.8 Hz, 1H), 4.03 (br. s., 1H), 3.69 (s, 3H), 2.79 (s, 3H).

LCMS found 435.01, [M+H]+.

6-(1H-indol-5-yl)-4-((3-methoxyphenyl)amino)-8-methylquinoline-3-carboxamide (141), synthesized from 120 using the General procedure E, yellow solid, Yield: 4%). $^1$H NMR (400 MHz, CD$_3$OD) $\delta$ 8.90 (s, 1H), 7.93 (d, J = 8.0 Hz, 2H), 7.54 (s, 1H), 7.34 (d, J = 8.8 Hz, 1H), 7.22 - 7.28 (m, 2H), 7.13 - 7.18 (m, 1H), 6.74 - 6.80 (m, 1H), 6.63 - 6.67 (m, 2H), 6.43 (d, J = 2.9 Hz, 1H), 3.73 (s, 3H), 2.80 (s, 3H). LCMS found 423.01, [M+H]$^+$.  

6-(3-(1H-tetrazol-5-yl)phenyl)-4-((3-methoxyphenyl)amino)-8-methylquinoline-3-carboxamide (142), synthesized from 120 using the General procedure E, light yellow solid, Yield: 2%). $^1$H NMR (400 MHz, CD$_3$OD) $\delta$ 8.90 (s, 1H), 8.28 (s, 1H), 8.10 (s, 1H), 8.02 (s, 1H),
7.98 (d, J = 8.0 Hz, 1H), 7.45 (t, J = 7.7 Hz, 1H), 7.35 (d, J = 8.0 Hz, 1H), 7.24 (t, J = 8.0 Hz, 1H), 6.66 - 6.73 (m, 3H), 3.71 (s, 3H), 2.83 (s, 3H), 2.66 (s, 3H). LCMS found 452.01, [M+H]^+.

6-(benzo[d][1,3]dioxol-5-yl)-4-((3-methoxyphenyl)amino)-8-methylquinoline-3-carboxamide (143), (synthesized from 120 using the General procedure E, light yellow solid, Yield: 12%). 1H NMR (400 MHz, DMSO-d6) δ 10.41 (s, 1H), 8.96 (s, 1H), 8.21 (br. s., 1H), 7.85 (s, 1H), 7.79 (s, 1H), 7.67 (br. s., 1H), 7.17 (t, J = 8.0 Hz, 1H), 6.90 - 6.97 (m, 3H), 6.62 - 6.66 (m, 1H), 6.60 (s, 1H), 6.53 (d, J = 7.3 Hz, 1H), 6.03 (s, 2H), 3.67 (s, 3H), 2.72 (s, 3H). LCMS found 428.01, [M+H]^+.

6-(3-acetylphenyl)-4-((3-methoxyphenyl)amino)-8-methylquinoline-3-carboxamide (144), (synthesized from 120 using the General procedure E, yellow solid, Yield: 35%). 1H NMR (400 MHz, DMSO-d6) δ 10.51 (s, 1H), 9.01 (s, 1H), 8.26 (br. s., 1H), 7.88 - 8.00 (m, 4H), 7.17
(d, J = 6.6 Hz, 2H), 7.52 - 7.62 (m, 1H), 7.18 (t, J = 8.0 Hz, 1H), 6.61 - 6.68 (m, 2H), 6.55 (d, J = 8.0 Hz, 1H), 3.68 (s, 3H), 2.77 (s, 3H), 2.57 (s, 3H). LCMS found 426.01, [M+H]^+.

6-(1H-indazol-6-yl)-4-(3-methoxyphenylamino)-8-methylquinoline-3-carboxamide, formate salt (145), (synthesized from 120 using the General procedure E, yellow solid, Yield: 14%). 1H NMR (500 MHz, DMSO-d6) δ 13.15 (br. s., 1H), 10.45 (s, 1H), 8.99 (s, 1H), 8.23 (br. s., 1H), 8.19 (s, 1H), 8.07 (s, 1H), 7.95 (s, 1H), 7.97 (s, 1H), 7.75 (d, J = 8.3 Hz, 1H), 7.69 (br. s., 1H), 7.56 (s, 1H), 7.19 (t, J = 8.0 Hz, 1H), 7.16 (d, J = 8.8 Hz, 1H), 6.67 (dd, J = 1.9, 8.3 Hz, 1H), 6.61 (s, 1H), 6.57 (d, J = 7.8 Hz, 1H), 3.68 (s, 3H), 2.77 (s, 3H). LCMS found 424.01, [M+H]^+.

methyl2-(3-carbamoyl-4-((3-methoxyphenyl)amino)-8-methylquinolin-6-yl)benzoate, formate salt (146), (synthesized from 120 using the General procedure E, yellow solid, Yield: 26%). 1H NMR (500 MHz, DMSO-d6) δ 10.27 (s, 1H), 9.00 (s, 1H), 8.22 (br. s., 1H), 8.13 (s,
methyl 4-(3-carbamoyl-4-((3-methoxyphenyl)amino)-8-methylquinolin-6-yl)benzoate (147),
(synthesized from 120 using the General procedure E, light yellow solid, Yield: 20%). $^1$H
NMR (500 MHz, DMSO-$d_6$) $\delta$ 10.46 (s, 1H), 8.98 (s, 1H), 8.21 (br. s., 1H), 7.93 - 8.00 (m, 4H),
7.68 (br. s., 1H), 7.59 (d, $J = 8.8$ Hz, 2H), 7.18 (t, $J = 8.0$ Hz, 1H), 6.67 (dd, $J = 1.9, 8.3$ Hz, 1H),
6.63 (s, 1H), 6.57 (d, $J = 7.8$ Hz, 1H), 3.85 (s, 3H), 3.68 (s, 3H), 2.75 (s, 3H). LCMS found
442.01, [M+H]$^+$. 

\begin{center}
\includegraphics[width=0.5\textwidth]{image.png}
\end{center}
4-((3-methoxyphenyl)amino)-8-methyl-6-(3-(trifluoromethoxy)phenyl)quinoline-3-carboxamide (148), (synthesized from 120 using the General procedure E, yellow solid, Yield: 35%). ¹H NMR (500 MHz, DMSO-\(d_6\)) \(\delta\) 10.57 (s, 1H), 9.02 (s, 1H), 8.27 (br. s., 1H), 7.96 (s, 1H), 7.90 (s, 1H), 7.73 (br. s., 1H), 7.53 - 7.57 (m, 2H), 7.32 - 7.37 (m, 1H), 7.26 (s, 1H), 7.17 - 7.23 (m, 1H), 6.65 - 6.71 (m, 2H), 6.57 (d, \(J = 7.8\) Hz, 1H), 3.69 (s, 3H), 2.76 (s, 3H). LCMS found 468.01, [M+H]+.

dimethyl 5-(3-carbamoyl-4-((3-methoxyphenyl)amino)-8-methylquinolin-6-yl)isophthalate (149), (synthesized from 120 using the General procedure E, yellow solid, Yield: 52%). ¹H NMR (500 MHz, DMSO-\(d_6\)) \(\delta\) 10.58 (s, 1H), 9.02 (s, 1H), 8.42 (s, 1H), 8.27 (br. s., 1H), 8.18 (d, \(J = 1.5\) Hz, 2H), 7.98 (s, 1H), 7.94 (s, 1H), 7.73 (br. s., 1H), 7.20 (t, \(J = 8.0\) Hz, 1H), 6.70 (dd, \(J\)
= 1.9, 8.3 Hz, 1H), 6.64 (s, 1H), 6.56 (d, J = 7.8 Hz, 1H), 3.92 (s, 6H), 3.69 (s, 3H), 2.77 (s, 3H).

LCMS found 500.01, [M+H]^+.

3-(3-carbamoyl-4-((3-methoxyphenyl)amino)-8-methylquinolin-6-yl)-5-(methoxycarbonyl) benzoic acid (150), (synthesized from 120 using the General procedure E, white solid, Yield: 12%).  1H NMR (500 MHz, DMSO-\(d_6\)) \(\delta\) 10.50 (s, 1H), 9.00 (s, 1H), 8.42 (s, 1H), 8.24 (d, J = 7.3 Hz, 2H), 8.04 (br. s., 1H), 7.95 (s, 2H), 7.69 (br. s., 1H), 7.17 (t, J = 8.0 Hz, 1H), 6.66 (dd, J = 1.9, 8.3 Hz, 1H), 6.62 (s, 1H), 6.55 (d, J = 7.8 Hz, 1H), 3.90 (s, 3H), 3.68 (s, 3H), 2.77 (s, 3H).

LCMS found 486.01, [M+H]^+.

5-(3-carbamoyl-4-((3-methoxyphenyl)amino)-8-methylquinolin-6-yl)isophthalic acid (151), (synthesized from 120 using the General procedure E, white solid, Yield: 25%).  1H NMR (500 MHz, DMSO-\(d_6\)) \(\delta\) 13.25 (br. s., 1H), 10.47 (s, 1H), 10.47 (s, 1H), 8.99 (s, 1H), 8.39 (s, 1H), 8.21 (s, 1H), 8.15
(m, 2H), 7.96 (s, 2H), 7.67 (s, 1H), 7.15 (t, \(J = 8.0\) Hz, 1H), 6.62-6.56 (m, 2H), 6.54 (d, \(J = 7.0\) Hz, 1H), 3.68 (s, 3H), 2.77 (s, 3H). LCMS found 472.01, [M+H]+.

4-((3-methoxyphenyl)amino)-8-methyl-6-(3-(oxazol-2-yl)phenyl)quinoline-3-carboxamide (152), (synthesized from 120 using the General procedure E, yellow solid, Yield: 10%). \(^1\)H NMR (500 MHz, DMSO-\(d_6\)) \(\delta\) 10.52 (s, 1H), 9.00 (s, 1H), 8.29 (s, 1H), 8.25 (br. s., 1H), 7.92 - 7.99 (m, 2H), 7.71 (br. s., 1H), 7.60 - 7.63 (m, 1H), 7.54 - 7.59 (m, 1H), 7.42 (s, 1H), 7.20 (t, \(J = 8.0\) Hz, 1H), 6.70 (dd, \(J = 2.2, 8.0\) Hz, 1H), 6.64 (s, 1H), 6.57 (d, \(J = 7.8\) Hz, 1H), 3.67 (s, 3H), 2.77 (s, 3H). LCMS found 451.01, [M+H]+.

4-((3-methoxyphenyl)amino)-8-methyl-6-(3-(5-methyl-1,3,4-oxadiazol-2-yl)phenyl)quinoline-3-carboxamide, formate salt (153), (synthesized from 120 using the General procedure E, yellow solid, Yield: 16%). \(^1\)H NMR (500 MHz, DMSO-\(d_6\)) \(\delta\) 10.52 (s, 1H), 9.02 (s, 1H), 8.24 -
8.31 (m, 2H), 7.92 - 8.04 (m, 4H), 7.69 - 7.75 (m, 2H), 7.65 (d, \(J = 7.3\) Hz, 1H), 7.21 (t, \(J = 8.0\) Hz, 1H), 6.64 - 6.70 (m, 2H), 6.58 (d, \(J = 8.3\) Hz, 1H), 3.69 (s, 3H), 2.79 (s, 3H), 2.64 (s, 3H).

LCMS found 466.01, [M+H]\(^+\).

4-((3-methoxyphenyl)amino)-8-methyl-6-(3-(5-methyl-1,3,4-thiadiazol-2-yl)phenyl)quinoline-3-carboxamide, formate salt (154), (synthesized from 120 using the General procedure E, yellow solid, Yield: 13%). \(^1\)H NMR (500 MHz, DMSO-\(d_6\)) \(\delta\) 10.50 (s, 1H), 9.01 (s, 1H), 8.28 (s, 1H), 8.25 (br. s., 1H), 7.99 (s, 1H), 7.95 (s, 1H), 7.90 (d, \(J = 7.8\) Hz, 1H), 7.86 (s, 1H), 7.71 (br. s., 1H), 7.64 (d, \(J = 7.8\) Hz, 1H), 7.58 (t, \(J = 7.8\) Hz, 1H), 7.18 (t, \(J = 8.0\) Hz, 1H), 6.64 - 6.68 (m, 2H), 6.54 (d, \(J = 8.3\) Hz, 1H), 3.70 (s, 3H), 2.81 (s, 3H), 2.77 (s, 3H). LCMS found 482.01, [M+H]\(^+\).
3-(3-carbamoyl-4-((3-methoxyphenyl)amino)-8-methylquinolin-6-yl)benzoic acid (155), (synthesized from 120 using the General procedure E, yellow solid, Yield: 13%). $^1$H NMR (500 MHz, DMSO-$d_6$) $\delta$ 10.49 (s, 1H), 9.01 (s, 1H), 8.25 (br. s., 1H), 8.01 (s, 1H), 7.95 (s, 2H), 7.91 (d, $J = 7.8$ Hz, 1H), 7.67 - 7.74 (m, 2H), 7.54 (t, $J = 7.8$ Hz, 1H), 7.19 (t, $J = 7.8$ Hz, 1H), 6.62 - 6.68 (m, 2H), 6.57 (d, $J = 8.3$ Hz, 1H), 3.70 (s, 3H), 2.78 (s, 3H). LCMS found 428.01, [M+H]$^+$. 

4-((3-methoxyphenyl)amino)-8-methyl-6-(3-(3-methyl-1,2,4-oxadiazol-5-yl)phenyl)quinoline-3-carboxamide, formate salt (156), (synthesized from 120 using the General procedure E, yellow solid, Yield: 18%). $^1$H NMR (500 MHz, DMSO-$d_6$) $\delta$ 10.51 (s, 1H), 9.00 (s, 1H), 8.31 (s, 1H), 8.23 (br. s., 1H), 8.03-8.05 (m, 2H), 7.99 (s, 1H), 7.96 (s, 1H), 7.79 (d, $J = 8.0$ Hz, 1H), 7.69 (br. s., 1H), 7.67 (t, $J = 8.5$ Hz, 1H), 7.19 (t, $J = 8.2$ Hz, 1H), 6.64-6.67 (m, 1H), 6.63 (s, 1H), 6.58 (d, $J = 7.5$ Hz, 1H), 3.68 (s, 3H), 2.77 (s, 3H), 2.45 (s, 3H). LCMS found 466.01, [M+H]$^+$. 

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methyl-3-(3-carbamoyl-4-((3-methoxyphenyl)amino)quinolin-6-yl)benzoate (157), (synthesized from 106 using the General procedure E, yellow solid, Yield: 20%). ¹H NMR (500 MHz, DMSO-d₆) δ 10.62 (s, 1H), 8.98 (s, 1H), 8.25 (br. s., 1H), 8.07 (s, 1H), 8.00 - 8.05 (m, 2H), 7.90 - 7.95 (m, 2H), 7.75 (d, J = 7.8 Hz, 1H), 7.69 (br. s., 1H), 7.55 - 7.60 (m, 1H), 7.21 (t, J = 8.0 Hz, 1H), 6.70 (d, J = 8.3 Hz, 1H), 6.67 (s, 1H), 6.59 (d, J = 7.3 Hz, 1H), 3.88 (s, 3H), 3.69 (s, 3H). LCMS found 428.01, [M+H]⁺.

4-((3-methoxyphenyl)amino)-6-(1-methyl-1H-indazol-6-yl)quinoline-3-carboxamide (158), (synthesized from 106 using the General procedure E, yellow solid, Yield: 13%). ¹H NMR (400 MHz, DMSO-d₆) δ 10.72 (s, 1H), 9.00 (s, 1H), 8.29 (br. s., 1H), 8.11 - 8.17 (m, 1H), 8.09 (s, 1H), 7.99 - 8.05 (m, 2H), 7.76 (d, J = 8.8 Hz, 1H), 7.71 (br. s., 1H), 7.52 (s, 1H), 7.19 - 7.29 (m, 2H), 6.70 - 6.76 (m, 2H), 6.62 (d, J = 8.0 Hz, 1H), 4.03 (s, 3H), 3.70 (s, 3H). LCMS found 424.01, [M+H]⁺.
methyl 3-(3-carbamoyl-8-ethyl-4-((3-methoxyphenyl)amino)quinolin-6-yl)benzoate **(159)**, (synthesized from **106** using the **General procedure E**, yellow solid, Yield: 34%). $^1$H NMR (500 MHz, CD$_3$OD) $\delta$ 8.93 (s, 1H), 7.97 (s, 1H), 7.89 - 7.95 (m, 2H), 7.82 (s, 1H), 7.59 (d, $J$ = 7.8 Hz, 1H), 7.45 (t, $J$ = 7.6 Hz, 1H), 7.21 (t, $J$ = 8.0 Hz, 1H), 6.74 (dd, $J$ = 1.9, 7.8 Hz, 1H), 6.64 (t, $J$ = 1.9 Hz, 1H), 6.60 (d, $J$ = 7.8 Hz, 1H), 3.93 (s, 3H), 3.71 (s, 3H), 3.27 (q, $J$ = 7.5 Hz, 2H), 1.40 (t, $J$ = 7.5 Hz, 3H). LCMS found 456.01, [M+H]$^+$. 

8-ethyl-4-((3-methoxyphenyl)amino)-6-(1-methyl-1H-indazol-6-yl)quinoline-3-carboxamide **(160)**, (synthesized from **106** using the **General procedure E**, yellow solid, Yield: 45%). $^1$H NMR (500 MHz, DMSO-$d_6$) $\delta$ 10.58 (s, 1H), 9.01 (s, 1H), 8.28 (br. s., 1H), 8.03 (s, 1H), 7.99 (s, 1H), 7.93 - 7.96 (m, 1H), 7.74 (d, $J$ = 8.3 Hz, 1H), 7.72 (br. s., 1H), 7.52 (s, 1H), 7.19 - 7.25 (m,
2H), 6.66 - 6.72 (m, 2H), 6.57 (d, J = 7.8 Hz, 1H), 4.03 (s, 3H), 3.69 (s, 3H), 3.27 (q, J = 7.3 Hz, 2H), 1.37 (t, J = 7.5 Hz, 3H). LCMS found 452.01, [M+H]^+.

**methyl 3-(3-carbamoyl-8-methyl-4-(pyridin-3-ylamino)quinolin-6-yl)benzoate (161),** (synthesized from 106 using the General procedure E, yellow solid, Yield: 12%). \(^1\)H NMR (400 MHz, CD3OD) \(\delta\) 8.95 (s, 1H), 8.34 (d, J = 2.2 Hz, 1H), 8.27 (d, J = 3.6 Hz, 1H), 8.08 (s, 1H), 7.97 - 8.02 (m, 2H), 7.95 (s, 1H), 7.77 (d, J = 7.3 Hz, 1H), 7.53 (t, J = 8.0 Hz, 1H), 7.48 (d, J = 8.8 Hz, 1H), 7.38 (dd, J = 5.1, 8.0 Hz, 1H), 3.94 (s, 3H), 2.84 (s, 3H). LCMS found 413.01, [M+H]^+.

**4-((3,5-dichloropyridin-4-yl)amino)-8-methyl-6-(1-methyl-1H-indazol-6-yl)quinoline-3-carboxamide (162),** (synthesized from 106 using the General procedure E, yellow solid, Yield: 17%). \(^1\)H NMR (500 MHz, DMSO-\(d_6\)) \(\delta\) 9.08 (s, 1H), 8.76 (s, 2H), 8.61 (s, 1H), 8.51 (br. s., 2H),
8.12 - 8.17 (m, 2H), 8.08 (s, 1H), 8.10 (s, 1H), 7.87 (d, \( J = 8.3 \text{ Hz}, 1\text{H} \)), 7.69 - 7.73 (m, 1H), 4.14 (s, 3H), 2.75 (s, 4H). LCMS found 477.01, [M+H]⁺.

![Structure of compound 163](image)

Methyl 3-(3-carbamoyl-8-methyl-4-((3-methylbenzyl)amino)quinolin-6-yl)benzoate (163), (synthesized from 106 using the General procedure E, white solid, Yield: 40%). \(^1\)H NMR (500 MHz, CDCl₃) \( \delta \) 9.76 (t, \( J = 6.1 \text{ Hz}, 1\text{H} \)), 8.79 (s, 1H), 8.22 (s, 1H), 8.14 - 8.18 (m, 1H), 7.96 - 8.01 (m, 1H), 7.82 (s, 1H), 7.38 - 7.41 (m, 2H), 7.27 - 7.29 (m, 1H), 7.22 - 7.26 (m, 2H), 7.13 (d, \( J = 7.3 \text{ Hz}, 1\text{H} \)), 5.95 (br. s., 2H), 4.93 (d, \( J = 6.3 \text{ Hz}, 2\text{H} \)), 3.96 (s, 3H), 2.79 (s, 3H), 2.33 (s, 3H). LCMS found 440.03, [M+H]⁺.

![Structure of compound 164](image)

Methyl 3-(3-carbamoyl-4-((3-methoxybenzyl)amino)-8-methylquinolin-6-yl)benzoate (164), (synthesized from 106 using the General procedure E, white solid, Yield: 34%). \(^1\)H NMR (500 MHz, CDCl₃) \( \delta \) 9.82 (t, \( J = 6.1 \text{ Hz}, 1\text{H} \)), 8.81 (s, 1H), 8.21 (s, 1H), 8.15 (s, 1H), 7.95 - 8.01 (m, 1H), 7.82 (s, 1H), 7.37 - 7.44 (m, 2H), 7.30 (t, \( J = 7.8 \text{ Hz}, 1\text{H} \)), 7.03 (d, \( J = 7.3 \text{ Hz}, 1\text{H} \)), 6.96 (s,
1H), 6.85 (dd, J = 1.9, 8.3 Hz, 1H), 5.95 (br. s., 2H), 4.94 (d, J = 6.3 Hz, 2H), 3.96 (s, 3H), 3.76 (s, 3H), 2.79 (s, 3H). LCMS found 456.01, [M+H]^+.

methyl3-(3-carbamoyl-4-((3-methoxyphenyl)(methyl)amino)-8-methylquinolin-6-yl)benzoate (165), (synthesized from 106 using the General procedure E, yellow solid, Yield: 7%). \(^1\)H NMR (500 MHz, CD3OD) \(\delta\) 9.08 (s, 1H), 8.13 (s, 1H), 8.00 (d, J = 7.8 Hz, 1H), 7.97 (s, 1H), 7.84 - 7.88 (m, 1H), 7.76 (d, J = 7.8 Hz, 1H), 7.54 (t, J = 7.8 Hz, 1H), 7.10 (t, J = 8.3 Hz, 1H), 6.45 (dd, J = 1.7, 8.0 Hz, 1H), 6.31 (s, 1H), 6.27 (d, J = 8.3 Hz, 1H), 3.94 (s, 3H), 3.70 (s, 3H), 3.47 (s, 3H), 2.88 (s, 3H). LCMS found 456.01, [M+H]^+.

4-((2-methoxyphenyl)amino)-8-methyl-6-(1-methyl-1H-indazol-6-yl)quinoline-3-carboxamide, formate salt (166), (synthesized from 106 using the General procedure E, light yellow solid, Yield: 8%). \(^1\)H NMR (500 MHz, DMSO-d6) \(\delta\) 10.73 (s, 1H), 9.03 (s, 1H), 8.36 (br.
s., 1H), 8.16 (s, 1H), 8.01 (s, 1H), 7.99 (s, 1H), 7.75 - 7.78 (m, 1H), 7.73 (br. s., 1H), 7.70 (d, \(J = 8.3\) Hz, 1H), 7.39 (s, 1H), 7.17 (d, \(J = 3.9\) Hz, 2H), 7.08 - 7.12 (m, 1H), 6.90 (td, \(J = 4.2, 8.0\) Hz, 1H), 6.85 (d, \(J = 7.8\) Hz, 1H), 4.01 (s, 3H), 2.76 (s, 3H). LCMS found 438.01, [M+H]+.

**methyl 3-(4-(benzylamino)-3-carbamoyl-8-methylquinolin-6-yl)benzoate (167),** (synthesized from 106 using the **General procedure E**, white solid, Yield: 10%). 1H NMR (400 MHz, CDCl₃) \(\delta\) 9.80 (br. s., 1H), 8.81 (s, 1H), 8.21 (s, 1H), 8.17 (s, 1H), 7.98 (d, \(J = 5.8\) Hz, 1H), 7.82 (s, 1H), 7.36 - 7.47 (m, 6H), 7.29 - 7.35 (m, 1H), 4.98 (d, \(J = 6.6\) Hz, 2H), 3.96 (s, 3H), 2.80 (s, 3H). LCMS found 426.01, [M+H]+.

**methyl 3-((4-(benzylamino)-3-carbamoyl-8 -methylquinolin-6-yl)sulfonyl)benzoate (168),** (synthesized from 106 using an analogous procedure to product 122, yellow solid, Yield: 55%). 1H NMR (400 MHz, CD₃OD) \(\delta\) 8.83 (s, 1H), 8.78 (s, 1H), 8.50 (s, 1H), 8.23 (d, \(J = 7.3\) Hz, 1H), 8.00 (d, \(J = 8.0\) Hz, 1H), 7.96 (s, 1H), 7.66 (t, \(J = 7.7\) Hz, 1H), 7.30 - 7.43 (m, 5H), 4.92 (s, 2H), 3.92 (s, 3H), 2.68 (s, 3H). LCMS found 490.01, [M+H]+.
8-methyl-6-(1-methyl-1H-indazol-6-yl)-4-(phenylamino)quinoline-3-carboxamide   (169),
(synthesized from 106 using the General procedure E, yellow solid, Yield: 16%). $^1$H NMR
(500 MHz, DMSO-$d_6$) $\delta$ 10.73 (s, 1H), 9.03 (s, 1H), 8.31 (br. s., 1H), 8.01 (s, 2H), 7.83 - 7.88
(m, 1H), 7.74 (br. s., 1H), 7.71 (d, $J = 8.3$ Hz, 1H), 7.45 (s, 1H), 7.35 (t, $J = 7.8$ Hz, 2H), 7.12 -
7.16 (m, 2H), 7.07 (d, $J = 7.8$ Hz, 2H), 4.02 (s, 3H), 2.77 (s, 3H). LCMS found 408.01, [M+H]$^+$. 

4-((3-ethoxyphenyl)amino)-8-methyl-6-(1-methyl-1H-indazol-6-yl)quinoline-3-carboxamide
(170), (synthesized from 106 using the General procedure E, yellow solid, Yield: 22%). $^1$H NMR
(500 MHz, DMSO-$d_6$) $\delta$ 10.62 (s, 1H), 9.02 (s, 1H), 8.29 (br. s., 1H), 8.14 (s, 1H), 8.02 (d, $J = 3.9$ Hz, 2H), 7.93 (s, 1H), 7.71 - 7.76 (m, 2H), 7.51 (s, 1H), 7.18 - 7.23 (m, 2H), 6.69 (dd, $J = 1.9$, 8.3 Hz, 1H), 6.64 (s, 1H), 6.58 (d, $J = 7.8$ Hz, 1H), 4.03 (s, 3H), 3.94 (q, $J = 7.0$ Hz, 2H), 2.77 (s, 3H), 1.22 (t, $J = 6.8$ Hz, 3H). LCMS found 452.01, [M+H]$^+$. 

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8-methyl-6-(1-methyl-1H-indazol-6-yl)-4-(m-tolylamino)quinoline-3-carboxamide (171), (synthesized from 106 using the General procedure E, yellow solid, Yield: 20%). $^1$H NMR (500 MHz, DMSO-$d_6$) $\delta$ 10.70 (s, 1H), 9.01 (s, 1H), 8.29 (br. s., 1H), 8.00 (d, $J$ = 7.3 Hz, 2H), 7.89 (s, 1H), 7.72 (d, $J$ = 8.3 Hz, 2H), 7.42 (s, 1H), 7.22 (t, $J$ = 7.8 Hz, 1H), 7.16 (d, $J$ = 8.3 Hz, 1H), 6.96 (d, $J$ = 7.8 Hz, 1H), 6.93 (s, 1H), 6.83 (d, $J$ = 7.8 Hz, 1H), 4.01 (s, 3H), 2.76 (s, 3H), 2.25 (s, 3H). $^{13}$C NMR (126 MHz, DMSO-$d_6$) $\delta$ 170.8, 150.2, 149.4, 148.7, 144.2, 140.7, 139.5, 138.4, 138.2, 136.5, 132.9, 130.6, 129.8, 124.8, 123.5, 122.6, 122.4, 121.9, 120.6, 120.5, 119.0, 112.8, 107.9, 36.0, 21.7, 19.0. LCMS found 422.01, [M+H]$^+$.

4-(4-methoxy-3-methylphenylamino)-8-methyl-6-(1-methyl-1H-indazol-6-yl)quinoline-3-carboxamide, formate salt (172), (synthesized from 106 using the General procedure E, yellow solid, Yield: 22%). $^1$H NMR (500 MHz, DMSO-$d_6$) $\delta$ 10.93 (s, 1H), 8.98 (s, 1H), 8.28 (br. s., 1H), 8.15 (s, 1H), 8.02 (s, 1H), 7.96 (s, 1H), 7.86 (s, 1H), 7.70 (d, $J$ = 8.3 Hz, 2H), 7.34
(s, 1H), 7.11 (d, J = 8.3 Hz, 1H), 6.99 - 7.03 (m, 1H), 6.90 - 6.96 (m, 2H), 3.98 (s, 3H), 3.80 (s, 3H), 2.74 (s, 3H), 2.13 (s, 3H). LCMS found 452.01, [M+H]⁺.

4-((4-methoxyphenyl)amino)-8-methyl-6-(1-methyl-1H-indazol-6-yl)quinoline-3-carboxamide (173), (synthesized from 106 using the General procedure E, yellow solid, Yield: 18%). ¹H NMR (500 MHz, DMSO-d₆) δ 10.92 (s, 1H), 8.99 (s, 1H), 8.29 (br. s., 1H), 8.02 (s, 1H), 7.97 (s, 1H), 7.81 - 7.86 (m, 1H), 7.71 (d, J = 8.3 Hz, 2H), 7.37 (s, 1H), 7.10 - 7.13 (m, 1H), 7.08 (d, J = 8.8 Hz, 2H), 6.98 (d, J = 8.8 Hz, 2H), 4.00 (s, 3H), 3.77 (s, 3H), 2.75 (s, 3H). LCMS found 438.01, [M+H]⁺.

4-(3-ethylphenylamino)-8-methyl-6-(1-methyl-1H-indazol-6-yl)quinoline-3-carboxamide (174), (synthesized from 106 using the General procedure E, yellow solid, Yield: 9%). ¹H NMR (500 MHz, DMSO-d₆) δ 10.75 (s, 1H), 9.02 (s, 1H), 8.30 (br. s., 1H), 8.02 (s, 2H), 7.88 (s, 1H), 7.73 (br. s., 1H), 7.71 (d, J = 8.3 Hz, 1H), 7.46 (s, 1H), 7.27 (t, J = 7.8 Hz, 1H), 7.10 - 7.14
(m, 1H), 7.01 (d, \( J = 7.3 \) Hz, 1H), 6.93 (s, 1H), 6.88 (d, \( J = 7.8 \) Hz, 1H), 4.01 (s, 3H), 2.77 (s, 3H), 2.54 (q, 2H), 1.07 (t, \( J = 7.3 \) Hz, 3H). LCMS found 436.01, [M+H]⁺.

**Procedure for the synthesis of intermediates 110 a and 110b**

4-chloro-6-iodo-N,8-dimethylquinoline-3-carboxamide (110a). To sodium hydride (60 % dispersion in mineral oil, 10.9 mg, 0.27 mmol) in DMF (2 mL) was added intermediate 110 (100 mg, 0.289 mmol) in dry DMF (3 mL). The reaction mixture was stirred for 35 minutes at rt before adding iodomethane (0.017 mL, 0.274 mmol), after which the reaction mixture was stirred at rt overnight. Then, to the reaction was added water and EtOAc. The aqueous layer was extracted with EtOAc (3x). The combined organics were washed with brine and finally dried under sodium sulfate. The solvent was concentrated and the product was confirmed by LCMS, LCMS found 360.01, [M+H]⁺. The product was taken to the next step without further characterization.

4-chloro-6-iodo-N,N,8-trimethylquinoline-3-carboxamide (110b). To sodium hydride (60 % dispersion in mineral oil, 23 mg, 0.57 mmol) in DMF (2 mL) was added intermediate 110 (100 mg, 0.289 mmol) in dry DMF (3 mL). The reaction mixture was stirred for 35 minutes at rt
before adding iodomethane (0.020 mL, 0.317 mmol), after which the reaction mixture was stirred at rt overnight. Then, to the reaction mixture was added water and EtOAc. The aqueous layer was extracted with EtOAc (3x). The combined organics were washed with brine and finally dried under sodium sulfate. The solvent was concentrated and the product was confirmed by LCMS, LCMS found 374.01, [M+H]^+. The product was taken to the next step without further characterization.

**General procedure F.**

Compound 110a or 110b (1 equiv) were dissolved in acetonitrile and 3-methoxyaniline (1.1 equiv) was added. The mixture was heated and refluxed overnight. The precipitate formed was filtered, washed with acetonitrile and the solid obtained was dried under high vacuum to give 106a or 106b analogs.

![Chemical structures](image)
4-((3-methoxyphenyl)amino)-N,8-dimethyl-6-(1-methyl-1H-indazol-6-yl)quinoline-3-carboxamide (175) (synthesized using General procedure E from 106a, yellow solid, Yield: 8%). $^1$H NMR (500 MHz, DMSO-$d_6$) $\delta$ 10.05 (s, 1H), 8.87 (s, 1H), 8.59 (d, $J$ = 4.4 Hz, 1H), 8.14 (s, 1H), 8.07 (s, 1H), 8.06 (s, 1H), 7.80 (d, $J$ = 8.3 Hz, 1H), 7.69 (s, 1H), 7.34 - 7.37 (m, 1H), 7.21 (t, $J$ = 8.0 Hz, 1H), 6.64 - 6.68 (m, 2H), 6.58 (d, $J$ = 8.3 Hz, 1H), 4.07 (s, 3H), 3.70 (s, 3H), 2.80 (s, 3H), 2.66 (d, $J$ = 4.4 Hz, 3H). LCMS found 452.01, [M+H]$^+$. 

4-((3-methoxyphenyl)amino)-N,N,8-trimethyl-6-(1-methyl-1H-indazol-6-yl)quinoline-3-carboxamide, formate salt (176), (synthesized using General procedure E from 106b, yellow solid, Yield: 17%). $^1$H NMR (500 MHz, DMSO-$d_6$) $\delta$ 9.11 (s, 1H), 8.55 (s, 1H), 8.51 (s, 1H), 8.13 (s, 2H), 8.07 (s, 1H), 8.04 (s, 1H), 7.86 (d, $J$ = 8.3 Hz, 1H), 7.66 (d, $J$ = 8.3 Hz, 1H), 7.17 (t, $J$ = 8.5 Hz, 1H), 6.63 (d, $J$ = 8.3 Hz, 1H), 6.55 - 6.59 (m, 2H), 4.12 (s, 3H), 3.71 (s, 3H), 2.79 (s, 3H), 2.75 (s, 3H), 2.38 (s, 3H). LCMS found 466.01, [M+H]$^+$. 

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6-(3-((1-ethyl-1H-pyrazol-5-yl)carbamoyl)phenyl)-4-((3-methoxyphenyl)amino)-8-methylquinoline-3-carboxamide, formate salt (177). To **155** (25.0 mg, 0.058 mmol) in DMF was added HATU (24.4 mg, 0.064 mmol). After 5 minutes 1-ethyl-1H-pyrazol-5-amine (13.0 mg, 0.117 mmol) and DIEA (0.021 ml, 0.123 mmol) were added. The resulting solution was stirred at room temperature overnight. The solvent was concentrated. The crude product was purified via preparative HPLC to give the product as a yellow solid (Yield: 30%). $^1$H NMR (500 MHz, DMSO-$d_6$) $\delta$ 10.41 and 10.47 (2s, 1H, rotamers), 10.33 (s, 1H), 8.99 (s, 1H), 8.22 (br. s., 1H), 8.10 - 8.15 (m, 2H), 8.00 (d, $J$ = 8.8 Hz, 2H), 7.88 - 7.95 (m, 1H), 7.66 - 7.73 (m, 1H), 7.61 (d, $J$ = 7.8 Hz, 1H), 7.55 (dd, $J$ = 8.0, 15.8 Hz, 1H), 7.44 (d, $J$ = 1.9 Hz, 1H), 7.12 - 7.19 (m, 1H), 6.57 - 6.66 (m, 2H), 6.55 (d, $J$ = 7.8 Hz, 1H), 6.23 (s, 1H), 4.02 (q, $J$ = 7.1 Hz, 2H), 3.66 and 3.68 (2s, 3H, rotamers), 2.76 and 2.78 (2s, 3H, rotamers), 1.31 (t, $J$ = 7.08 Hz, 3H). LCMS found 521.01, [M+H]$^+$. 

![Chemical Structure](image-url)
6-(3-((3,5-dimethylisoxazol-4-yl)carbamoyl)phenyl)-4-((3-methoxyphenyl)amino)-8-methylquinoline-3-carboxamide, formate salt (178), (synthesized from 155 similarly to 177, yellow solid, Yield: 35%). $^1$H NMR (500 MHz, DMSO-$d_6$) $\delta$ 10.41 (s, 1H), 9.86 (s, 1H), 8.98 (s, 1H), 8.19 - 8.24 (m, 2H), 8.10 (s, 1H), 7.99 (d, $J$ = 7.3 Hz, 2H), 7.90 (d, $J$ = 7.3 Hz, 1H), 7.68 (br. s., 1H), 7.57 - 7.62 (m, 1H), 7.55 (d, $J$ = 7.8 Hz, 1H), 7.14 (t, $J$ = 8.0 Hz, 1H), 6.60 - 6.63 (m, 1H), 6.58 (dd, $J$ = 1.95, 8.3 Hz, 1H), 6.53 (d, $J$ = 7.8 Hz, 1H), 3.65 (s, 3H), 2.77 (s, 3H), 2.31 (s, 3H), 2.13 (s, 3H). LCMS found 522.01, [M+H]$^+$. 

To 110 (200 mg, 0.577 mmol) was added (3-(methoxycarbonyl)phenyl)boronic acid (104 mg, 0.577 mmol), [1,1'bis(diphenylphosphino)ferrocene]dichloropalladium (II) complex with
dichloromethane (84 mg, 0.115 mmol), dioxane (4 mL) and sodium carbonate (1.73 mL, 3.46 mmol). The reaction mixture was run in the M.W. at 150 °C for 10 minutes. After the reaction mixture cooled to ambient temperature the crude was filtered through celite washing with MeOH/DCM (1:9). The filtrate was concentrated under reduced pressure. The crude mixture was chromatographed using hexanes/EtOAc to give as the major product 114 as a white solid (Yield: 29%) and a mixture of 111 and 112. This mixture was separated using preparative HPLC to give the clean products 111 (white solid, Yield: 5%) and 112 (white solid, Yield: 1%).

![Structure of dimethyl 3,3'-(3-carbamoyl-8-methylquinoline-4,6-diyl)dibenzoate (111)](image)

**dimethyl 3,3'-(3-carbamoyl-8-methylquinoline-4,6-diyl)dibenzoate** (111). $^1$H NMR (500 MHz, DMSO-$d_6$) $\delta$ 9.00 (s, 1H), 8.07 - 8.12 (m, 3H), 7.99 (s, 1H), 7.95 (d, $J = 7.8$ Hz, 1H), 7.83 - 7.89 (m, 2H), 7.69 - 7.75 (m, 2H), 7.60 (t, $J = 7.8$ Hz, 1H), 7.54 (s, 1H), 7.48 - 7.50 (m, 1H), 3.32 (s, 6H), 2.87 (s, 3H). LCMS found 455.11, [M+H]$^+$. 
methyl 3-(3-carbamoyl-8-methylquinolin-6-yl)benzoate (112). $^1$H NMR (500 MHz, CDCl$_3$) δ 9.31 (s, 1H), 8.69 (d, $J = 2.4$ Hz, 1H), 8.40 (s, 1H), 8.09 (d, $J = 7.8$ Hz, 1H), 7.97 (d, $J = 7.3$ Hz, 2H), 7.91 (d, $J = 7.8$ Hz, 1H), 7.59 (t, $J = 7.5$ Hz, 1H), 3.98 (s, 3H), 2.91 (s, 3H). LCMS found 321.01, [M+H]$^+$. 

methyl 3-(3-carbamoyl-4-chloro-8-methylquinolin-6-yl)benzoate (114). $^1$H NMR (500 MHz, DMSO-$d_6$) δ 8.90 (s, 1H), 8.30 - 8.35 (m, 2H), 8.21 (s, 1H), 8.13 - 8.17 (m, 2H), 8.03 (d, $J = 7.8$ Hz, 1H), 7.99 (s, 1H), 7.70 (t, $J = 7.8$ Hz, 1H), 3.91 (s, 3H), 2.82 (s, 3H). LCMS found 354.01, [M+H]$^+$. 

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methyl 3-(3-carbamoyl-4-(cyclopentylamino)-8-methylquinolin-6-yl)benzoate, formate salt (179). To 114 (8.70 mg, 0.025 mmol) was added acetonitrile and lastly cyclopentanamine (0.015 ml, 0.147 mmol) was added. The reaction was run at 80°C in a sealed vial for 48 h. The solvent was concentrated and the crude product was purified via preparative HPLC to give the desired product as a yellow solid (Yield: 38%). \(^1\)H NMR (500 MHz, CD\(_3\)OD) \(\delta\) 8.65 (s, 1H), 8.44 (s, 2H), 8.34 (s, 1H), 8.04 (d, \(J = 7.8\) Hz, 1H), 7.94 - 7.98 (m, 2H), 7.62 (t, \(J = 7.8\) Hz, 1H), 4.63 (t, \(J = 4.8\) Hz, 1H), 3.96 (s, 3H), 2.73 (s, 3H), 2.14 - 2.24 (m, 2H), 1.73 - 1.91 (m, 6H). LCMS found 404.14, [M+H]^+.

4-(cyclopentyl)oxy)-6-iodo-8-methylquinoline-3-carboxamide (180a). To 110 (100 mg, 0.289 mmol) in acetonitrile (4 mL) was added DBU (0.174 mL, 1.154 mmol) and cyclopentanol (0.159 mL, 1.731 mmol). The reaction was run in the M.W. for 40 min at 150 °C. The solvent was concentrated. The crude product was chromatographed using 0-6% MeOH in DCM to give the desired product (Yield: 20%). \(^1\)H NMR (500 MHz, CDCl\(_3\)) \(\delta\) 9.32 (s, 1H), 8.33 (s, 1H), 7.87 (s,
1H), 7.16 (br. s., 1H), 6.27 (br. s., 1H), 4.94 (m, 1H), 2.74 (s, 3H), 1.95 - 2.01 (m, 2H), 1.88 - 1.93 (m, 2H), 1.83 (m, 2H), 1.68 - 1.74 (m, 2H).

methyl 3-(3-carbamoyl-4-(cyclopentyloxy)-8-methylquinolin-6-yl)benzoate (180). To 180a (25.00 mg, 0.063 mmol) was added (3-(methoxycarbonyl)phenyl)boronic acid (45.4 mg, 0.252 mmol) and [1,1′-bis(diphenylphosphino)ferrocene]dichloropalladium (II) complex with dichloromethane (4.62 mg, 6.31 µmol), dioxane (2 mL) and sodium carbonate (0.189 ml, 0.379 mmol). The reaction was run in the M.W. at 120 °C for 20 minutes. Then, the reaction was cooled to ambient temperature, and the mixture was diluted with MeOH/DCM (1:9) and filtered through celite. The solvent was concentrated. The crude product was purified via reverse phase column chromatography 0-100% Acetonitrile in Water to give the desired product as a white solid (Yield: 15%). 

$^1$H NMR (500 MHz, CDCl₃) δ 9.40 (s, 1H), 8.40 (s, 1H), 8.20 (d, $J = 1.5$ Hz, 1H), 8.09 (d, $J = 7.8$ Hz, 1H), 7.86 - 7.94 (m, 2H), 7.60 (t, $J = 7.6$ Hz, 1H), 7.21 (br. s., 1H), 5.88 (br. s., 1H), 5.03 (td, $J = 2.9$, 5.5 Hz, 1H), 3.99 (s, 3H), 2.89 (s, 3H), 2.03 - 2.11 (m, 2H), 1.92 - 1.98 (m, 2H), 1.82 - 1.90 (m, 2H), 1.73 (t, $J = 7.3$ Hz, 2H).
4-chloro-8-methyl-6-(3-(oxazol-2-yl)phenyl)quinoline-3-carboxamide (113). To 110 (100 mg, 0.289 mmol) was added (3-(oxazol-2-yl)phenyl)boronic acid (49.1 mg, 0.260 mmol) and [1,1′-bis(diphenylphosphino)ferrocene]dichloropalladium (II) complex with dichloromethane (21.11 mg, 0.029 mmol), dioxane (3 mL) and sodium carbonate (0.866 ml, 1.731 mmol). The reaction was run in the M.W. at 120 °C for 20 minutes. The reaction was cooled to ambient temperature, and the mixture was diluted with MeOH/DCM (1:9) and filtered through celite. The solvent was concentrated. The crude was chromatographed using 0-90 % EtOAc in hexane to give the desired product (Yield: 14%). \(^1\)H NMR (500 MHz, CDCl\(_3\)) \(\delta\) 9.16 (s, 1H), 8.44 (s, 1H), 8.40 - 8.42 (m, 1H), 8.11 (d, \(J = 7.8\) Hz, 1H), 8.02 (s, 1H), 7.83 (d, \(J = 7.8\) Hz, 1H), 7.78 (s, 1H), 7.63 (t, \(J = 7.8\) Hz, 1H), 7.30 (s, 1H), 6.41 (br. s., 1H), 6.02 (br. s., 1H), 2.90 (s, 3H).

4-(cyclopentylamino)-8-methyl-6-(3-(oxazol-2-yl)phenyl)quinoline-3-carboxamide (181).

To 113 (13.00 mg, 0.036 mmol) was added acetonitrile and lastly cyclopentanamine (0.021 ml, 0.214 mmol). The reaction was run at 80°C in a sealed vial for 48 h. The solvent was
concentrated and the crude product was chromatographed using 0-7% MeOH in DCM to give the desired product as a yellow solid (Yield: 34%). $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 9.50 (d, $J = 7.3$ Hz, 1H), 8.75 (s, 1H), 8.39 (d, $J = 4.8$ Hz, 2H), 8.07 (d, $J = 7.8$ Hz, 1H), 7.87 (s, 1H), 7.76 (s, 1H), 7.75 (d, $J = 7.8$ Hz, 1H), 7.59 (t, $J = 7.6$ Hz, 1H), 7.29 (s, 1H), 5.90 (br. s., 2H), 4.55 - 4.64 (m, 1H), 2.80 (s, 3H), 2.11 - 2.20 (m, 2H), 1.78 - 1.90 (m, 4H), 1.68 - 1.75 (m, 2H). LCMS found 413.01, [M+H]$^+$.

4-(cyclopentyloxy)-8-methyl-6-(3-(oxazol-2-yl)phenyl)quinoline-3-carboxamide (182). To 113 (40.0 mg, 0.110 mmol) in acetonitrile (3 mL), cyclopentanol (0.060 mL, 0.660 mmol) and DBU (0.099 mL, 0.660 mmol). The reaction was run in the M.W. for 40 min at 150 °C. The solvent was concentrated. The crude product was purified via reverse phase column chromatography 0-100% Acetonitrile in Water to give the desired product as a white solid (Yield: 9%). $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 9.40 (s, 1H), 8.41 (s, 1H), 8.21 - 8.23 (m, 1H), 8.10 (d, $J = 7.8$ Hz, 1H), 7.96 (s, 1H), 7.77 - 7.81 (m, 2H), 7.62 (t, $J = 7.8$ Hz, 1H), 7.30 (s, 1H), 7.22 (br. s., 1H), 5.85 (br. s., 1H), 4.97 - 5.06 (m, 1H), 2.89 (s, 3H), 2.03 - 2.12 (m, 2H), 1.91 - 1.99 (m, 2H), 1.86 (dd, $J = 5.9$, 13.18 Hz, 2H), 1.72 (t, $J = 7.3$ Hz, 2H).
Appendix 3

Supplemental Biology Assay and Computational data for chapter 2
A) Biology assays for Chapter 2

**Cell Viability Screen**

*Cell Titre Blue® (CTB)*

Inhibitor sets were dissolved in DMSO and stored at -80°C as a 500x dilution series. The final concentrations ranged from 50 nM to 8 µM, and DMSO was constant at 0.2%. Each drug was tested at a minimum of 8 concentrations in triplicate. Log phase BF trypanosomes were counted with a hemocytometer and seeded at an initial density of $1 \times 10^5$ cells mL$^{-1}$ in a volume of 1.5mL in a 24 well plate format. The cultures were incubated with the inhibitor sets for 4-doubling times (48 hr). At the end of this time, trypanosomes were concentrated by centrifugation, suspended in 100 µl of medium and transferred to 96-well flat bottom microtiter plates (Costar UV transparent plates). The end-point CTB assay was used to test for cell viability (Promega). CTB reagent (20 µL) was added to each well and the plates were incubated at 37°C and 5% CO$_2$ for 3 hours. Fluorescence was measured at 560$_{Ex}$/590$_{Em}$ using the SPECTRAMax GEMINI XPS Microplate Spectrofluorometer (Molecular Devices). Background was calculated from wells containing CTB and media without cells, while 100% growth was calculated from cells with DMSO and without drugs. The background fluorescence was subtracted from all the data points. The EC$_{50}$ values were computed from the inhibition curves with OriginPro 8.5. The CTB end-point assay measures non-selective dehydrogenases, where the total activity is proportional to cell number. The CTB end-point assay was linear out to 180 min and at trypanosome cell densities in the range of $5 \times 10^5$-$2 \times 10^7$ cells mL$^{-1}$. 

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**Resazurin assay**

Cells were seeded at a final density of $1 \times 10^5$ cells/ml in 200 µl of HMI-9 medium on a 96-well plate (Costar plastic). Drug dilutions ranged from 100 nM to 10 µM, and eight drug concentrations were used. Each concentration was tested in triplicate. Separate control wells contained cells with DMSO only (the maximum readout; 6 wells) and culture medium without cells (the background readout; 6 wells). The DMSO concentration was 0.05%. Cells were cultured for 48 hr. At the end of this time, 20 µl of 0.2 mM resazurin (Sigma Cat #R7017) was added to each well. The cells were then grown for an additional 24 hr. Then, fluorescence was measured at $560_{\text{Ex}}/590_{\text{Em}}$ using the SPECTRAmax GEMINI XPS Microplate Spectrofluorometer (Molecular Devices) with a cut off filter of 570 nM.

**Biological testing of AT-9283 in mice**

All the *in vivo* work was performed by the research team from Southern Methodist University under the supervision of Professor Larry Ruben. The protocols with mice were approved by the Institutional Animal Care and Use Committee (IACUC). Briefly, the mice were first injected with $2 \times 10^4$ parasites by intraperitoneal (ip) injection. The next day, the dosing regimen was begun. Control and treated groups each contained 5 mice. Each group received ip injections bid for 5 days of either 20 mg/kg AT-9283, or of vehicle without drug. The drug was dissolved in DMSO, then propyl-cyclodextrin and water following a procedure reported by Curry *et al.*

After 120 hours (5 days) the parasitemia was estimated in peripheral blood. The control mice had average parasitemia of $5.3 \pm 2.4 \times 10^8$ cells per mL of blood whereas the treated mice had an average parasitemia of $3.9 \pm 1.6 \times 10^7$ cells per mL of blood.
B ) Computational chemistry

Comparison of the docking score from Glide SP docking and EC$_{50}$ data from the cell proliferation assay (Table S1). We compared the cellular potency data for 6 ligands for which both EC$_{50}$ values of *T. b. b.* and *T. b. r.* had been obtained (1, 2, 11, 12, 14, 18) with their docking scores. Figure S1 shows the scatter plots of docking scores and EC$_{50}$ of these ligands. The EC$_{50}$ values of *T. b. r.* and docking score are linearly correlated with an $R^2 = 0.75$; while the EC$_{50}$ values of *T. b. b.* and docking score are correlated with an $R^2 = 0.72$. These plots shows that the predicted rank-ordering of ligands using docking was roughly aligned with the observed potency against trypanosome cells, and the docking scores show a correlation with the potency values.
Table S1. Dose-response experiments on the parallel array of analogs of danusertib, with Glide docking scores.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Manuscript compound number</th>
<th>R₁</th>
<th>Ar</th>
<th>T. b.b. EC₅₀(µM)</th>
<th>T.b.r. EC₅₀(µM)</th>
<th>MOLT-4 EC₅₀(µM)</th>
<th>Selectivity T.b.r./MOLT</th>
<th>Glide docking score</th>
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<tbody>
<tr>
<td>Danusertib</td>
<td>1</td>
<td>OMe</td>
<td>phenyl</td>
<td>0.6</td>
<td>0.15</td>
<td>0.15</td>
<td>1.0</td>
<td>-10.34</td>
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<tr>
<td>PHA-680632</td>
<td>2</td>
<td>--</td>
<td>2,6-diethylphenyl</td>
<td>4</td>
<td>1.25</td>
<td>0.22</td>
<td>0.18</td>
<td>-9.18</td>
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<tr>
<td>NEU327</td>
<td>8</td>
<td>H</td>
<td>1-naphthyl</td>
<td>nd</td>
<td>0.61</td>
<td>14.25</td>
<td>23</td>
<td>-9.4</td>
</tr>
<tr>
<td>NEU336</td>
<td>9</td>
<td>H</td>
<td>2,3,6-trifluorophenyl</td>
<td>nd</td>
<td>0.32</td>
<td>2.22</td>
<td>0.14</td>
<td>-9.45</td>
</tr>
<tr>
<td>NEU338</td>
<td>10</td>
<td>OMe</td>
<td>phenyl</td>
<td>nd</td>
<td>0.61</td>
<td>4.13</td>
<td>0.15</td>
<td>-9.45</td>
</tr>
<tr>
<td>NEU328</td>
<td>11</td>
<td>H</td>
<td>3-Cl-Ph</td>
<td>0.85</td>
<td>0.58</td>
<td>4</td>
<td>0.15</td>
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<td>NEU343</td>
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<td>NEU340</td>
<td>14</td>
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<tr>
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<td>H</td>
<td>2,5-dimethylphenyl</td>
<td>nd</td>
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<td>NEU341</td>
<td>16</td>
<td>H</td>
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<td>2</td>
<td>2.31</td>
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<tr>
<td>NEU339</td>
<td>17</td>
<td>H</td>
<td>3-(2-methylindolyl)</td>
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<td>1.16</td>
<td>1.03</td>
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<td>0.91</td>
<td>0.63</td>
<td>1.44</td>
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</tbody>
</table>

*d* indicates compounds tested as racemate
Figure S1 The docking scores and EC$_{50}$ of six newly synthesized compounds are plotted to their EC$_{50}$ values of *T. b. r* and EC$_{50}$ values of *T. b. b*. The rank ordering of compounds by the docking experiments was aligned with the observed potency against trypanosome cells, and the docking scores show the correlation with the potency values.
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Appendix 4. Representative NMR spectra from Chapter 2 and 3
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Compound 32

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null
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Compound 74a

500 MHz
CDCl₃
Compound 75

500 MHz

CDCl₃
Compound 93

500 MHz
DMSO- $d_5$
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Compound 101

400 MHz
DMSO-d₆
Chemical Shift (ppm)

Normalized Intensity

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