Antimalarial benzoheterocyclic 4-aminoquinolines: Structure-activity relationship, in vivo evaluation, mechanistic and bioactivation studies.

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ABSTRACT: A novel class of benzoheterocyclic analogues of amodiaquine designed to avoid toxic reactive metabolite formation was synthesized and evaluated for antiplasmodial activity against K1 (multidrug resistant) and NF54 (sensitive) strains of the malaria parasite *Plasmodium*

falciparum. Structure-activity relationship studies led to the identification of highly promising analogs, the most potent of which had IC_{50}s in the nanomolar range against both strains. The compounds further demonstrated good in vitro microsomal metabolic stability while those subjected to in vivo pharmacokinetic studies had desirable pharmacokinetic profiles. In vivo antimalarial efficacy in *Plasmodium berghei* infected mice was evaluated for four compounds, all of which showed good activity following oral administration. In particular, compound **19** completely cured treated mice at a low multiple dose of 4×10 mg/kg. Mechanistic and bioactivation studies suggest hemozoin formation inhibition and a low likelihood of forming quinone-imine reactive metabolites, respectively.

KEYWORDS: amodiaquine, benzoxazole, antiplasmodial activity, antimalarial activity, malaria, reactive metabolite, 4-aminoquinolines; bioactivation; structure-activity relationship; β-hematin; quinone imine.

1. INTRODUCTION

Malaria remains a leading cause of morbidity and mortality globally. In 2012, there were an estimated 207 million cases of malaria and 627 000 deaths worldwide, with 90% of all malaria deaths occurring in sub-Saharan Africa.[1] One of the biggest challenges facing malaria chemotherapy is the rapid emergence of resistance to existing antimalarial drugs.[2] This challenge underscores the need for the continued search for new antimalarials.

Chloroquine (**1**) (structure shown in Figure 1), was undoubtedly one of the most successful antimalarials ever owing to its good efficacy and low cost which made it affordable especially in

the developing countries with high malaria endemicity.[3] Chloroquine was replaced as first line therapy by the sulfonamide antimalarials and, later on, artemisinin combination therapy (ACT), following the development of widespread resistance against the drug by *Plasmodium falciparum*.[4] An aromatic side chain analogue of chloroquine, amodiaquine (**2**), however, retains activity against chloroquine-resistant *Plasmodium* strains.[5] Besides, it is an established fact that resistance against these 4-aminoquinolines is not a result of target modification but is caused by impaired accumulation of the drug at the target.[6,7] Consequently, amodiaquine is an attractive lead compound in the search for new antimalarials. Despite the desirable antimalarial efficacy of amodiaquine, chronic use especially during prophylaxis has been found to precipitate severe hepatotoxicity, myelotoxicity and agranulocytosis.[8,9] This toxicity has been attributed to the bioactivation of amodiaquine to reactive quinone imine (**3**) and aldehyde quinone imine (**4**) metabolites (figure 1) which covalently bind to cellular macromolecules causing drug-induced toxicity and cell damage directly or via immunological mechanisms.[10–12]

<<figure 1>>

The avoidance of amodiaquine bioactivation has been the subject of a number of previous studies. Park and co-workers have demonstrated that the 4′-hydroxyl group of amodiaquine could be replaced with a 4′-fluorine atom to produce 4'-dehydroxy-4'-fluoroamodiaquine (**5**) with antimalarial activity in the low nanomolar range.[11,13] Miroshnikova and coworkers synthesized various isotebuquine analogs (**6**) with excellent antimalarial activity but poor oral bioavailability.[14] The most successful campaign towards circumventing amodiaquine bioactivation was the synthesis of isoquine (**7**) and its analogues by O'Neill and co-workers.[15] One of these analogues, *N-tert*-butyl isoquine (**8**), progressed to clinical trials but its development has been discontinued due to exposures insufficient to demonstrate drug safety superior to chloroquine.[16–18] Figure 2

shows the chemical structures of some of the compounds synthesized to circumvent amodiaquine bioactivation.

<<figure 2>>

In a previous paper, we reported for the first time the synthesis and potent antiplasmodial activity of benzothiazolyl (**9**-**13**), benzimidazolyl (**14**-**17**), benzoxazolyl (**18**-**19**) and pyridyl (**20**-**21**) analogues of amodiaquine (figure 3) designed to prevent bioactivation to both the quinone imine and aldehyde metabolites.[19] The present paper reports on the bioactivation studies on these compounds and the selection, expanded synthesis and structure activity relationship (SAR) studies of the benzoxazole analogues. The in vitro antiplasmodial, mechanistic and metabolic stability evaluation as well as in vivo pharmacokinetic and efficacy studies on this class of compounds is presented.

<<figure 3>>

Bioactivation potential can be evaluated using various approaches including covalent binding studies and trapping with different reagents following incubation with microsomes or hepatocytes.[20] The trapping reagents used include glutathione (for soft electrophiles such as the quinone imine) as well as methoxylamine and potassium cyanide (for hard electrophiles such as the iminium ion).[21–23] Electrochemical oxidation online with electrospray ionization mass spectrometry (EC-ESI/MS) is a relatively new technique that avoids the complexity of working with biological matrices. Electrochemical oxidation has been found to successfully mimic CYP450 benzylic hydroxylation, hydroxylation of aromatic rings containing electron-donating groups, N-dealkylation, S-oxidation, dehydrogenation and, less efficiently, N-oxidation and O-dealkylation.[24] Johansson and co-workers have demonstrated the utility of electrochemical

oxidation to mimic CYP450 and liver microsome catalyzed oxidation of amodiaquine and desethylamodiaquine.[25] Other researchers have employed electrochemistry to mimic phase I oxidation of paracetamol, clozapine, trimethoprim and diclofenac.[26,27] The coupling of EC with MS means that MS/MS can be used to provide important structural information about the reactive metabolites formed.

2. RESULTS AND DISCUSSION

2.1. Bioactivation studies on previously synthesized analogues

The bioactivation potential of our previously synthesized analogues **9-21** was evaluated using trapping experiments involving glutathione, potassium cyanide and methoxylamine. The EC-ESI/MS system was utilized whereby mass spectra were scanned for the presence of adduct peaks arising from covalent bond formation between any reactive intermediates produced (upon oxidative bioactivation) and the trapping agents.

The results of the glutathione trapping experiment were semi-quantitatively expressed as the ratio of the sum of test compound adduct peak areas to clozapine adduct peak area and were represented graphically (figure 4). Clozapine is commonly used as a model compound that undergoes bioactivation to a nitrenium intermediate which, in turn, readily forms a glutathione conjugate.[28,29] A value of zero implies that no adduct was detected for the test compound while a value greater than one implies the sum of adduct peak areas for the test compound is greater than the area of the clozapine adduct peak. This implies that the compound has a higher potential for bioactivation than clozapine. Our study demonstrated that benzoxazole analogues **18** and **19** had the lowest potential for bioactivation to the quinone imine metabolite.[30] Benzothiazole analogues **9**, **11** and **12** had the largest glutathione ratios (0.74, 0.88 and 1.13, respectively) and hence the highest

potential for bioactivation to soft electrophilic metabolites. The proposed bioactivation mechanisms, facilitated by the more nucleophilic nitrogen and sulphur atoms, to quinone-like intermediates for the benzothiazolyl, benzimidazolyl and pyridyl series are shown in figure 5.

<<figure 4>>

<<figure 5>>

Results for the trapping of hard electrophiles using potassium cyanide and methoxylamine were expressed as positive or negative depending on the detection or lack thereof, respectively, of cyanide and methoxylamine adduct peaks in the spectra of the test compounds (figure 4). The benzothiazole *tert*-butyl side chain analogue **9**, the benzimidazole ethyl side chain analogue **14** and the benzoxazole ethyl side chain analogue **18** did not undergo trapping with either reagent. We concluded that trapping was purely dependent on the nature of the side chain, such that only molecules with a highly basic tertiary amine on the side chain could form reactive species capable of reacting with potassium cyanide and methoxylamine. Such an observation can be explained using the well known metabolic pathway for tertiary amines which involves formation of iminium ions and aldehydes.[31] Our previous results (table 1) had shown that a highly basic tertiary amine side chain gave the most potent antiplasmodial activity. Thus a functional group considered essential for activity was also involved in bioactivation. However, the aldehyde metabolite of amodiaquine is conjugated with the aminophenol ring and, therefore, relatively stable. The aldehyde metabolite formed by these compounds is attached to the benzoxazole nucleus via a labile amino group. The low bioactivation potential of the benzoxazole series in the glutathione trapping experiment made this series attractive for further exploration.

<<Table 1>>

2.2. Chemistry

The synthesis of novel benzoxazoles **22**-**41** starting with 2-amino-4-nitrophenol, carried out as previously described, is shown in scheme 1.[19] Novel analogues **42** and **43** were synthesized by a one step coupling of appropriately substituted commercially available benzoxazole-5-amines with 4,7-dichloroquinoline.

<<scheme 1>>

2.3. In vitro antiplasmodial activity

The two previously reported benzoxazole analogues (**18** and **19**) and the newly synthesized analogues **22**-**43** were tested against the chloroquine-sensitive NF54 and the multidrug resistant K1 strains of *Plasmodium falciparum*. Results are presented in table 2. Selected intermediate compounds were tested alongside the target compounds and the results of these are presented in the Supporting Information (SI Table 1).

<<table 2>>

The most potent compounds **19**, **23**, **24**, **27**, **28** and **29** had IC_{50} values equal to or comparable to that of chloroquine (0.001 μM) against the chloroquine-sensitive NF54 strain (Table 2). These potent compounds were all dialkylamino or cycloalkylamino analogues with a highly basic tertiary nitrogen and could be classified as either ethylamino (**19**, **23**, **27** and **29**) or propylamino (**24**, **26**, **28** and **30**) linker analogues. Activity against the NF54 strain did not vary widely, ranging between 0.001 and 0.019 μM, except for the morpholino analogues. The ethylmorpholino analogue **25** was the least active compound against the NF54 strain among the highly basic tertiary nitrogen analogues (IC_{50} = 0.108 μM), followed by the propylmorpholino analogue **26** (IC_{50} = 0.039 μM).

Variations from which a tentative SAR could be derived were noticeable in K1 activity. Changing the dialkylamino group from diethyl to dimethyl did not have any effect on activity in ethylamino linker analogues as both **27** and **29** had an IC_{50} of 0.042 μM. Changing to a propylamino linker resulted in the diethyl analogue **28** (IC_{50} = 0.085 μM) being more potent than the dimethyl analogue **30** (IC_{50} = 0.149 μM). A similar trend was observed for the piperidine analogues where the ethylpiperidine compound **23** (IC_{50} = 0.024 μM) was more active than the propylpiperidine analogue **24** (IC_{50} = 0.039 μM). However, this trend was reversed among the morpholino analogues **25** and **26** mentioned earlier where the propylamino linker analogue was the more potent analogue. Converting the tertiary amine to an amide, as in **22**, resulted in a drastic decline in activity against both strains, more so against the NF54 (IC_{50} = 1.876 μM) than the K1 (IC_{50} = 0.773 μM) strain. These results underscored the importance of the highly basic nitrogen.

Further exploratory studies involved the synthesis of analogues in which the side chain was attached to the benzoxazole group via a carbon instead of a nitrogen atom (Table 2). Although analogue **42** had a highly basic nitrogen, it showed remarkably reduced activity against both strains (NF54 IC_{50} = 0.213 μM, K1 IC_{50} = 0.411 μM) compared to the alkylamino or cycloalkylamino analogues. The reduction in activity could not simply be accounted for by the low predicted pKa (7.75) since the morpholino analogues with even lower pKas showed better activity. Attachment to the benzoxazole ring via a carbon atom was, therefore, considered to adversely affect antiplasmodial activity. Analogue **43** only exhibited micromolar activity (NF54 IC_{50} = 1.094 μM, K1 IC_{50} = 2.111 μM), probably due to the presence of a carbon linker and the lack of a highly basic nitrogen.

When the alkylamino groups were replaced with substituted benzylamines (**31** and **32**), activity dropped to the low submicromolar range. The benzylamine analogues were less active than

chloroquine against the NF54 strain and virtually equipotent with the drug against the K1 strain. The methylpyridine analogues (**33**, **35** and **36**) had activity in the mid-submicromolar to low micromolar range with activity increasing in the order 4-methyl < 2-methyl < 3-methyl. Increasing the length of the linker from a methyl to an ethyl as in **34** did not affect activity when compared to the corresponding 2-methylpyridine analogue **33**. As with the rest of the analogues discussed above, these compounds were more active against the chloroquine-sensitive NF54 strain as compared to the multidrug-resistant K1 strain.

Among the piperazinyl linker analogues, the methylpiperazine analogue with the piperazine ring directly attached to the benzoxazole ring **39** was more active than the corresponding 1-amino-4-methylpiperazinyl analogue **37**. Indeed **39** retained activity against the multidrug-resistant K1 strain (IC_{50} = 0.099 μM). Attachment of aromatic groups to the piperazine linker (**40** and **41**) resulted in further loss of activity against both the NF54 and K1 strains. The chloro substituted phenyl ring in **41** resulted in a two-fold improvement in activity against the K1 strain (IC_{50} = 0.412 μM) over **36** with an unsubstituted phenyl ring (IC_{50} = 0.875 μM). In all cases, the pattern of sensitivity observed with the other analogues was maintained. In terms of structure, analogue **38** was a homologue of the diethylamino analogues **27** and **28**. The reduced activity of **38** shows the importance of the aminoalkyl linker in the antiplasmodial potency of these compounds.

Whereas all the intermediates tested were considered inactive (IC_{50}>20 μM), intermediate **19d** with a highly basic tertiary nitrogen showed low micromolar activity against both plasmodial strains (SI Table 1). Overall, these findings pointed to the fact that the quinoline ring was essential for antiplasmodial activity. Compounds with potent antiplasmodial activity exhibited good selectivity for *Plasmodium falciparum* over Chinese hamster ovary cells as seen from the high selectivity indexes in table 2.

2.3. β-Hematin inhibition studies

The two previously reported benzoxazole analogues (**18** and **19**) and the newly synthesized analogues **22-43** were subjected to the β-hematin inhibition assay using a previously described procedure[32] in an attempt to establish whether or not these compounds exert their antiplasmodial effect similarly to other 4-aminoquinolines. The results of the β-hematin inhibition assay (table 2) were correlated with the antiplasmodial activity against the NF54 strain in which resistance mechanisms that impair drug accumulation are not yet established. The cycloalkylamine compounds **19**, **23**, **24**, **25** and **26** had IC_{50} values of between 80.0 and 105.0 μM. The dialkylamine analogues **27-30** were less potent inhibitors of β-hematin formation (IC_{50}s = 165.0-244.2 μM). Ethyldialkylamine analogues **27** and **29** (IC_{50} = 244.2 and 363.2 μM, respectively) had lower activity compared to the propyldialkylamine analogues **28** and **30** (IC_{50} = 165.0 and 173.7 μM, respectively). Overall, there was a lack of correlation between β-hematin inhibition and antiplasmodial activity against the NF54 strain as illustrated by the observation that compounds with the most potent β-hematin inhibition did not exhibit the most potent antiplasmodial activity. This lack of correlation may imply that the compounds exert their antiplasmodial effect through additional mechanisms or that the compounds do not accumulate in the acidic food vacuole efficiently. The non-quinoline benzoxazole intermediates did not show any inhibitory activity even at the highest concentration of 1000 μM (data not tabulated). Thus it can be concluded, as expected, that the quinoline ring is essential for the β-hematin inhibition observed in this series of compounds.

A statistically significant linear correlation was found between the logP of the compound with the inverse of the β-hematin inhibition activity (data supplied in SI Table 2) (figure 6). An increase in logP was found to improve β-hematin inhibition activity according to the equation $1/\beta HIC_{50}$ =

0.0084(logP) – 0.02, r^2=0.65, $p < 0.0001$, statistically significant at the 95% confidence level. This correlation suggests that compounds with greater logP have higher potency for preventing the growth of β-hematin crystals. Such compounds have an additional aromatic phenyl ring on the benzoxazole end of the molecule. Furthermore, compounds with the highest inhibitory activity possess inductively electron withdrawing (EW) substituents on the phenyl ring (Cl, CF_3). This may contribute favourably to the widely accepted π-π-stacking phenomenon for quinolines, whereby the molecule prevents β-hematin crystallization growth via a π-π interaction with a face of the hemozoin crystal.[33] Hence, the extra ring, in particular one with an EW substituent, may strengthen the molecule's interaction with heme, resulting in increased activity. However, since analogues with an extra aromatic ring did not have a highly basic tertiary amine, they may not accumulate as efficiently as highly basic tertiary amines in the parasitic food vacuole where these compounds are believed to act.

<<figure 6>>

2.4. In vitro microsomal metabolic stability studies

Microsomal metabolic stability was evaluated using a single-time-point assay in which the percentage of parent compound remaining after 30 minutes incubation of the test compound in the presence of pooled human liver or mouse liver microsomes (HLMs or MLMs) and NADPH was determined.[34] The two previously reported benzoxazole analogues (**18** and **19**) and the newly synthesized analogues **22**-**43** were evaluated. Microsomal stability results are presented in figure 7. Ethyl side chain analogue **18** was extremely unstable in MLMs but very stable in HLMs. The most stable compound was the dimethylamino analogue **29**. This analogue was virtually not

metabolized in MLMs and HLMs after 30 minutes of incubation. Propylmorpholino analogue **26** was the least stable analogue with <30% remaining after 30 minutes of incubation with both MLMs and HLMs. Ethyldialkylamino analogues **27** and **29** were observed to be more metabolically stable than the corresponding propyldialkylamino analogues **28** and **30**, respectively. This may be construed to imply that the propyl linker is the source of metabolic lability. The methylpiperazine analogue **39** was moderately stable in HLMs but unstable in MLMs, with only 18 % of the compound remaining after 30 minutes. Except for **19** and **41**, the analogues were generally more stable in HLMs than in MLMs. Overall, benzoxazole analogues with potent antiplasmodial activity (**19**, **23**, **24**, **27** and **29**) exhibited metabolic stability equal to or greater than amodiaquine in HLMs. In MLM incubations, the analogues were at least twice as stable as amodiaquine except for **23** with only 41 % of the parent compound remaining after 30 minutes. It was apparent that in MLMs the metabolic stability of the cycloalkylamino compounds decreased in the order morpholine analogues > pyrrolidine analogues > piperidine analogues.

<<figure 7>>

2.5. In vivo pharmacokinetic studies

The pharmacokinetic evaluation of compound **24** was performed in C57Bl/6 mice following oral and intravenous administration, n = 3 over 24 hours. Linear and semi-log plots are shown in figure 8. Non-compartmental analysis was performed and the results given in table 3. Compound **24** had good bioavailability of 56% and was defined as having low clearance, assuming low clearance to be below a third of the average mouse liver blood flow of 90 mL/min/kg,[35] while the volume of distribution was high at 24.5 L/kg resulting in a long elimination half-life of approximately 10 hours. Compound **24** was absorbed at a moderate rate with T_{max} at approximately 3 hours. The high volume of distribution and low clearance suggests the compound accumulates in secondary

compartments and is slowly released back into blood, increasing *in vivo* efficacy and mean survival days. The low clearance observed *in vivo* for **24** is not consistent with the observed *in vitro* metabolic depletion rates for this series of compounds which may point to the role of other factors such as high microsomal and plasma protein binding affecting drug elimination.

<<figure 8>>

<<table 3>>

2.6. In vivo efficacy studies

The in vivo antimalarial efficacy of four of the most promising compounds was evaluated using a *P. berghei* mouse model by determining parasitemia reduction and mean survival days (MSD) for single- or multi-dose regimens. The results, following oral (p.o.) administration, for test compounds **19**, **23**, **24** and **29** as well as the antimalarial drugs chloroquine and amodiaquine, are summarized in table 4. At the highest oral doses (4×50 mg/kg), all the compounds displayed excellent parasitemia reduction (>99.8%). Compound **19**, which was among the initial sets of compounds synthesized, afforded complete cure with all six treated mice surviving through the entire 30 day period without any signs of toxicity. Five out of six mice treated with **24** were completely cured with all six mice attaining 30 mean survival days. Compound **29**, the dimethyl analog with good in vitro microsomal stability also exhibited potent activity achieving 29.3 mean survival days (2 out of 3 mice cured) while mice treated with **23** survived for an average of 27 days (1 out of 3 mice cured). The three best performing compounds were further tested at a single oral dose of 50 mg/kg and all compounds showed >99% parasitemia reduction. Compound **19** was the most potent (23.3 MSDs) while **24** (14.0 MSDs) and **29** (13.7 MSDs) were virtually equipotent.

When these three compounds were subjected to a low multi-dose oral regimen of 4 × 10 mg/kg, potent parasitemia reduction (>99%) was maintained. Remarkably, **19** afforded complete cure (>30 MSDs) at this low dose making it the most promising compound in this series. Compound **29** was the second most potent (24 MSDs, 1 out of 3 mice cured) while **24** (15.7 MSDs) gave the lowest survival rate.

<<table 4>>

3. CONCLUSION

A novel series of orally active antimalarial benzoxazole-4-aminoquinolines has been identified. The compounds in this series demonstrated potent in vitro antiplasmodial activity against both chloroquine-sensitive and drug-resistant *Plasmodium falciparum* strains and good in vivo efficacy in a murine malaria model. The compounds showed promising microsomal stability and an attractive pharmacokinetic profile. Preliminary mechanistic studies pointed to inhibition of β-hematin formation as one possible mechanism of action for these compounds, with the lack of strong correlation between inhibition and antiplasmodial activity suggesting that other mechanisms of action may be involved. The compounds circumvented bioactivation to the quinone imine and the relatively stable aldehyde metabolite of amodiaquine. Compound **19** combines good *in vitro* activity against *P. falciparum* with oral efficacy in a *P. berghei* mouse model.

4. EXPERIMENTAL

4.1. Chemistry

4.1.1 . General remarks

All commercially available chemicals were purchased from either Sigma-Aldrich or Combi-Blocks. All solvents were dried by appropriate techniques. Unless otherwise stated, all solvents used were anhydrous. ^{1}H NMR spectra were recorded on a Varian Mercury Spectrometer at 300 MHz or a Varian Unity Spectrometer at 400 MHz with Me_4Si as internal standard. ^{13}C NMR spectra were recorded at 75 MHz on a Varian Mercury Spectrometer or at 100 MHz on Varian Unity Spectrometer with Me_4Si as internal standard. Spectra were recorded at ambient temperature, unless otherwise stated. Chemical shifts (δ) are reported in parts per million downfield from Me_4Si and referenced to residual solvent. Standard abbreviations indicating multiplicity are used as follows: br s = broad, d = doublet, m = multiplet, q = quartet, quint. = quintet, s = singlet, t = triplet. Coupling constants, *J*, are recorded in Hertz (Hz). High resolution mass spectrometry (ESI) was performed using a Waters API Q-TOF Ultima instrument while low resolution mass spectrometry (LRMS) (EI^+) was performed on a JEOL GC Mate III spectrometer. Analytical thin-layer chromatography (TLC) was performed on aluminium-backed silica-gel 60 F_{254} (70-230 mesh) plates. Column chromatography was performed with Merck silica-gel 60 (70-230 mesh). Purity was determined by HPLC and all compounds were confirmed to have > 95% purity.

4.1.2 General procedure for the synthesis of chloroquinolinylbenzoxazolamines

Potassium ethyl xanthate (1872.4 mg, 11.7 mmol, 2 eq) was added to a solution of 2-amino-4-nitrophenol or 2-amino-4-chloronitrophenol (5.8 mmol, 1 eq) in 25 ml of absolute ethanol. The

reaction was heated at reflux for 4 h. The reaction mixture was then cooled to room temperature and concentrated to dryness under vacuum. The residue was dissolved in water and acidified to pH 5 using acetic acid. The resulting precipitate was filtered, washed with water and dried for 48 h to give the products which were used without further purification. Iodomethane (1.2 eq) and anhydrous potassium carbonate (1 eq) were added to a solution of 5-nitrobenzoxazole-2-thiol or 5-chlorobenzoxazole-2-thiol (1 eq) in acetonitrile (15 ml). The reaction mixture was stirred at room temperature for 4h. The reaction was quenched with a drop of water and the solvent removed under vacuum. The residue was suspended in water and extracted three times with ethyl acetate. The combined organic fractions were washed with brine (1×10ml) and dried over $MgSO_4$. Solvent evaporation under vacuum yielded the products which were used without further purification. A mixture of 2-bromo-5-nitrobenzothiazole, 2-bromo-5-nitro-1H-benzimidazole, 2-bromo-5-nitropyridine, 2-(methylthio)-5-nitrobenzoxazole, 5-chloro-2-(methylthio)-benzoxazole, (1 eq) and the appropriate amine (3 eq) in acetonitrile was heated in a microwave reactor at 120 °C for 20-30 min. The solvent was removed under vacuum and the residue dissolved in EtOAc, washed with water (2×10 ml), a solution of saturated $NaHCO_3$ (only employed when bromo-substituted reactants were used to neutralize HBr), brine (10 ml) and dried over $MgSO_4$. The solvent was removed under vacuum to obtain the products in crude mixture. Column chromatography using a MeOH: EtOAc (0-20%) (for tertiary amine products) or a hexane: EtOAc gradient afforded the pure products. The aromatic nitro-compound (1 mmol) was dissolved in ethanol (8 ml) with warming in a hydrogenator bottle. 10% Pd/C (0.3 times the weight of the compound being reduced) was added to this solution. The reaction bottle was sealed, filled with H_2 and evacuated twice. It was then shaken on a Parr shaker for 12 h at 60 psi of H_2 until completion of the reaction (monitored by TLC). The reaction mixture was filtered through Celite and the solvent removed

under vacuum to obtain the aromatic amine. The aromatic amine (1 mmol, 1 eq) was mixed with 4,7-dichloroquinoline (1.1 eq) in acetonitrile (25 ml). The mixture was acidified with 3-5 drops of HCl and stirred at reflux for 24 h. The solvent was removed under vacuum after which the residue was dissolved in EtOAc (or 20% methanol in dichloromethane for tertiary amine compounds) and washed with saturated $NaHCO_3$ (3×20 ml) and brine (1×10ml). The combined organic fractions were dried over anhydrous $MgSO_4$, adsorbed onto silica gel (5g) and subjected to silica gel column chromatography to afford the target compounds.

4.1.2.1. ***N^5-(7-Chloroquinolin-4-yl)-N^2-(2-(pyrrolidin-1-yl)ethyl)-benzoxazole-2,5-diamine,*** **19**

61 % yield. mp 98-102 °C. ^{1}H NMR (400 MHz, CD_3OD) δ 8.26 (d, J = 5.6 Hz, 1H), 8.21 (d, J = 9.0 Hz, 1H), 7.79 (d, J = 2.2 Hz, 1H), 7.40 (dd, J = 9.0, 2.2 Hz, 1H), 7.24 (d, J = 8.4 Hz, 1H), 7.20 (d, J = 2.1 Hz, 1H), 6.96 (dd, J = 8.4, 2.1 Hz, 1H), 6.70 (d, J = 5.6 Hz, 1H), 3.52 (t, J = 6.7 Hz, 2H), 2.74 (t, J = 6.7 Hz, 2H), 2.58 (m, 4H), 1.77 (m, 4H). ^{13}C NMR (101 MHz, CD_3OD) δ 163.56, 150.98, 150.62, 148.76, 146.07, 143.68, 135.85, 135.24, 126.40, 125.12, 123.18, 117.87, 117.19, 111.62, 108.76, 100.79, 54.72, 53.66, 41.21 (×2), 22.88 (×2). Anal. RP-HPLC t_R = 8.64 min (method D, purity 97%). HRMS (ESI+): *m/z* = 408.1577 (exact mass for $C_{22}H_{22}N_5OCl$ = 408.1591).

4.1.2.2. ***1-(3-(5-(7-Chloroquinolin-4-ylamino)benzoxazol-2-ylamino)propyl)pyrrolidin-2-one,*** **22**

65 % yield. mp 95-97 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.39 (d, J=5.5 Hz, 1H), 8.02 (d, J=9.0 Hz, 1H), 7.98 (d, J=2.1 Hz, 1H), 7.40 (dd, J=9.0, 2.1 Hz, 1H), 7.24 (d, J=2.0 Hz, 1H), 7.19 (d, J=8.4 Hz, 1H), 6.92 (dd, J=8.4, 2.0 Hz, 1H), 6.72 (d, J=5.5 Hz, 1H), 3.34 - 3.45 (m, 6H), 2.42 (t, J=8.1 Hz, 2H), 2.14 – 1.99 (m, 2H), 1.93 – 1.80 (m, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 176.16, 163.15, 150.70, 149.82, 148.38, 146.55, 144.51, 135.64, 135.04, 127.68, 125.94, 122.00, 117.54, 117.15, 112.39, 109.20, 101.52, 47.50, 39.57, 39.53, 30.89, 26.44, 17.95. Anal. RP-HPLC t_R =

9.95 min (method B, purity 97%). LRMS (EI+): *m/z* = 435.1 (exact mass for $C_{23}H_{22}ClN_5O_2$ = 435.1462).

4.1.2.3. *N^5-(7-Chloroquinolin-4-yl)-N^2-(2-(piperidin-1-yl)ethyl)benzoxazole-2,5-diamine,* 23

69 % yield. mp 89-91 °C. ^{1}H NMR (400 MHz, CD_3OD) δ 8.28 (d, *J*=5.6 Hz, 1H), 8.24 (d, *J*=9.0 Hz, 1H), 7.81 (d, *J*=2.2 Hz, 1H), 7.43 (dd, *J*=9.0, 2.2 Hz, 1H), 7.27 (d, *J*=8.4 Hz, 1H), 7.22 (d, *J*=2.1 Hz, 1H), 6.99 (dd, *J*=8.4, 2.2 Hz, 1H), 6.73 (d, *J*=5.6 Hz, 1H), 3.54 (t, *J*=6.7 Hz, 2H), 2.63 (t, *J*=6.7 Hz, 2H), 2.56 – 2.42 (m, 4H), 1.66 – 1.53 (m, 4H), 1.49 – 1.40 (m, 2H); ^{13}C NMR (101 MHz, CD_3OD) δ 163.59, 150.92, 150.68, 148.69, 146.09, 143.65, 135.85, 135.29, 126.35, 125.16, 123.21, 117.87, 117.24, 111.64, 108.80, 100.79, 57.46, 54.16, 39.34, 25.14, 23.68. Anal. RP-HPLC t_R = 8.79 min (method A, purity >99%). LRMS (EI+): *m/z* = 421.1 (exact mass for $C_{18}H_{17}N_3O_3S$ = 421.1669).

4.1.2.4. *N^5-(7-Chloroquinolin-4-yl)-N^2-(3-(piperidin-1-yl)propyl)benzoxazole-2,5-diamine,* 24

58 % yield. mp 87-89 °C. ^{1}H NMR (300 MHz, CD_3OD) δ 8.26 (d, *J*=5.6 Hz, 1H), 8.21 (d, *J*=9.0 Hz, 1H), 7.79 (d, *J*=2.2 Hz, 1H), 7.39 (dd, *J*=9.0, 2.2 Hz, 1H), 7.24 (d, *J*=8.4 Hz, 1H), 7.20 (d, *J*=2.1 Hz, 1H), 6.96 (dd, *J*=8.4, 2.1 Hz, 1H), 6.71 (d, *J*=5.6 Hz, 1H), 3.39 (t, *J*=6.8 Hz, 2H), 2.45 – 2.32 (m, 6H), 1.93 – 1.75 (m, 2H), 1.63 – 1.49 (m, 4H), 1.48 – 1.34 (m, 2H). ^{13}C NMR (101 MHz, CD_3OD) δ 163.66, 151.02, 150.77, 148.80, 146.08, 143.70, 135.89, 135.28, 126.40, 125.16, 123.22, 117.90, 117.28, 111.62, 108.77, 100.81, 56.38, 54.12, 41.03, 25.76, 25.18, 23.79. Anal. RP-HPLC t_R = 9.11 min (method A, purity >99%). LRMS (EI+): *m/z* = 435.1 (exact mass for $C_{24}H_{26}ClN_5O$ = 435.1826).

4.1.2.5. *N^5-(7-Chloroquinolin-4-yl)-N^2-(2-morpholinoethyl)benzoxazole-2,5-diamine,* 25

57 % yield. mp 107-109 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.27 (d, *J*=5.8 Hz, 1H), 8.07 (d, *J*=9.2 Hz, 1H), 7.96 (d, *J*=2.0 Hz, 1H), 7.36 (dd, *J*=9.2, 2.0 Hz, 1H), 7.20 (d, *J*=2.2 Hz, 1H), 7.16 (d, *J*=8.4 Hz, 1H), 6.92 (dd, *J*=8.4, 2.2 Hz, 1H), 6.65 (d, *J*=5.8 Hz, 1H), 3.57 – 3.74 (m, 4H), 3.49 (t, *J*=5.8 Hz, 2H), 2.61 (t, *J*=5.8 Hz, 2H), 2.43 – 2.50 (m, 4H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 163.00, 150.75, 149.81, 148.45, 146.48, 144.35, 135.59, 135.27, 127.59, 125.87, 122.10, 117.60, 117.28, 112.47, 109.23, 101.53, 66.84 (×2), 56.86, 53.30, 39.14 (×2). Anal. RP-HPLC t_R = 2.82 min (method C, purity >99%). LRMS (EI+): *m/z* = 423.0 (exact mass for $C_{22}H_{22}N_5O_2Cl$ = 423.1462).

4.1.2.6. *N^5-(7-Chloroquinolin-4-yl)-N^2-(3-morpholinopropyl)benzoxazole-2,5-diamine,* 26

53 % yield. mp 182-183 °C. ^{1}H NMR (300 MHz, CD_3OD) δ 8.28 (d, *J*=5.6 Hz, 1H), 8.23 (d, *J*=9.0 Hz, 1H), 7.81 (d, *J*=2.2 Hz, 1H), 7.42 (dd, *J*=9.0, 2.2 Hz, 1H), 7.26 (d, *J*=8.4 Hz, 1H), 7.21 (d, *J*=2.1 Hz, 1H), 6.98 (dd, *J*=8.4, 2.1 Hz, 1H), 6.72 (d, *J*=5.6 Hz, 1H), 3.74 – 3.62 (m, 4H), 3.53 (t, *J*=6.6 Hz, 2H), 3.43 (t, *J*=6.6 Hz, 2H), 2.51 – 2.37 (m, 4H), 1.91 – 1.78 (m, 2H). ^{13}C NMR (101 MHz, CD_3OD) δ 163.63, 150.96, 150.64, 148.73, 146.01, 143.70, 135.85, 135.25, 126.39, 125.14, 123.19, 117.88, 117.15, 111.55, 108.74, 100.80, 66.35, 56.04, 53.38, 40.88, 25.58. Anal. RP-HPLC t_R = 11.52 min (method A, purity >99%). LRMS (EI+): *m/z* = 437.1 (exact mass for $C_{23}H_{24}ClN_5O$ = 437.1619).

4.1.2.7. *N^5-(7-Chloroquinolin-4-yl)-N^2-(2-(diethylamino)ethyl)benzoxazole-2,5-diamine,* 27

61 % yield. mp 91-93 °C. ^{1}H NMR (300 MHz, CD_3OD) δ 8.27 (d, *J*=5.6 Hz, 1H), 8.23 (d, *J*=9.0 Hz, 1H), 7.80 (d, *J*=2.2 Hz, 1H), 7.42 (dd, *J*=9.0, 2.2 Hz, 1H), 7.27 (d, *J*=8.4 Hz, 1H), 7.21 (d, *J*=2.1 Hz, 1H), 6.98 (dd, *J*=8.4, 2.1 Hz, 1H), 6.72 (d, *J*=5.6 Hz, 1H), 3.49 (t, *J*=6.9 Hz, 2H), 2.73 (t, *J*=6.9 Hz, 2H), 2.61 (q, *J*=7.2 Hz, 4H), 1.05 (t, *J*=7.2 Hz, 6H). ^{13}C NMR (101 MHz, CD_3OD) δ 163.62, 150.97, 150.67, 148.75, 146.10, 143.71, 135.87, 135.26, 126.39, 125.14, 123.20, 117.89, 117.23, 111.66, 108.78, 100.80, 51.44, 46.78, 39.97, 10.29. Anal. RP-HPLC t_R = 11.34 min (method A, purity >99%). LRMS (EI+): *m/z* = 409.2 (exact mass for $C_{22}H_{24}ClN_5O$ = 409.1669).

4.1.2.8. *N^5-(7-Chloroquinolin-4-yl)-N^2-(3-(diethylamino)propyl)benzoxazole-2,5-diamine,* 28

46 % yield. mp 79-80 °C. ^{1}H NMR (400 MHz, CD_3OD) δ 8.25 (d, *J*=5.6 Hz, 1H), 8.21 (d, *J*=9.0 Hz, 1H), 7.78 (d, *J*=2.2 Hz, 1H), 7.37 (dd, *J*=9.0, 2.2 Hz, 1H), 7.23 (d, *J*=8.4 Hz, 1H), 7.20 (d, *J*=2.2 Hz, 1H), 6.95 (dd, *J*=8.4, 2.2 Hz, 1H), 6.70 (d, *J*=5.6 Hz, 1H), 3.40 (t, *J*=6.8 Hz, 2H), 2.68

– 2.45 (m, 6H), 1.92 – 1.71 (m, 2H), 1.02 (t, *J*=7.2 Hz, 6H). ^{13}C NMR (101 MHz, CD_3OD) δ 163.58, 150.95, 150.53, 148.73, 145.96, 143.65, 135.83, 135.21, 126.42, 125.11, 123.20, 117.87, 117.08, 111.49, 108.74, 100.78, 49.96, 46.46, 41.03, 25.60, 9.98. Anal. RP-HPLC t_R = 9.02 min (method A, purity >99%). LRMS (EI+): *m/z* = 423.2 (exact mass for $C_{23}H_{26}ClN_5O$ = 423.1826).

4.1.2.9. *N^5-(7-Chloroquinolin-4-yl)-N^2-(2-(dimethylamino)ethyl)benzoxazole-2,5-diamine,* 29

44 % yield. ^{1}H NMR (300 MHz, CD_3OD) δ 8.30 (d, *J*=5.6 Hz, 1H), 8.26 (d, *J*=9.0 Hz, 1H), 7.83 (d, *J*=2.2 Hz, 1H), 7.46 (dd, *J*=9.0, 2.2 Hz, 1H), 7.31 (d, *J*=8.4 Hz, 1H), 7.23 (d, *J*=2.2 Hz, 1H), 7.01 (dd, *J*=8.4, 2.2 Hz, 1H), 6.74 (d, *J*=5.6 Hz, 1H), 3.54 (t, *J*=6.6 Hz, 2H), 2.65 (t, *J*=6.6 Hz, 2H), 2.33 (s, 6H). ^{13}C NMR (101 MHz, CD_3OD) δ 163.61, 150.90, 150.80, 148.67, 146.16, 143.68, 135.86, 135.33, 126.31, 125.18, 123.23, 117.87, 117.35, 111.73, 108.82, 100.80, 57.72, 44.11, 39.96. Anal. RP-HPLC t_R = 8.56 min (method B, purity 99%). LRMS (EI+): *m/z* = 381.1 (exact mass for $C_{20}H_{20}ClN_5O$ = 381.1356).

4.1.2.10. *N^5-(7-Chloroquinolin-4-yl)-N^2-(3-(dimethylamino)propyl)benzoxazole-2,5-diamine,* 30

52 % yield. mp 55-56 °C. ^{1}H NMR (400 MHz, CD_3OD) δ 8.28 (d, *J*=5.6 Hz, 1H), 8.24 (d, *J*=9.0 Hz, 1H), 7.81 (d, *J*=2.2 Hz, 1H), 7.43 (dd, *J*=9.0, 2.2 Hz, 1H), 7.28 (d, *J*=8.4 Hz, 1H), 7.22 (d, *J*=2.0 Hz, 1H), 6.99 (dd, *J*=8.4, 2.1 Hz, 1H), 6.72 (d, *J*=5.6 Hz, 1H), 3.43 (t, *J*=6.9 Hz, 2H), 2.53 (t, *J*=7.7 Hz, 2H), 2.33 (s, 6H), 1.92 – 1.85 (m, 2H). ^{13}C NMR (101 MHz, CD_3OD) δ 163.64, 150.90, 150.71, 148.67, 146.06, 143.65, 135.84, 135.29, 126.32, 125.16, 123.23, 117.86, 117.25, 111.61, 108.79, 100.79, 56.42, 43.81 (×2), 40.55, 26.43. Anal. RP-HPLC t_R = 2.65 min (method C, purity 95%). LRMS (EI+): *m/z* = 395.1 (exact mass for $C_{21}H_{22}ClN_5O$ = 395.1513).

4.1.2.11. *N^2-(3-Chlorobenzyl)-N^5-(7-chloroquinolin-4-yl)benzoxazole-2,5-diamine,* 31

39 % yield. mp 98-99 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.50 (d, *J*=5.4 Hz, 1H), 8.04 (d, *J*=2.1 Hz, 1H), 7.91 (d, *J*=9.0 Hz, 1H), 7.46 (dd, *J*=9.0, 2.1 Hz, 1H), 7.41 (s, 1H), 7.34 – 7.23 (m, 5H), 7.00 (dd, *J*=8.4, 2.1 Hz, 2H), 6.79 (d, *J*=5.4 Hz, 1H), 4.70 (s, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ

162.75, 151.79, 149.63, 148.84, 146.65, 144.40, 139.63, 135.77, 135.28, 130.07, 128.98, 128.10, 127.75, 127.65, 125.92, 125.58, 121.06, 117.49, 112.87, 109.42, 102.10, 46.63. Anal. RP-HPLC t_R = 13.93 min (method A, purity >99%). LRMS (EI+): *m/z* = 434.0 (exact mass for $C_{23}H_{16}Cl_2N_4O$ = 434.0701).

4.1.2.12. *N^5-(7-Chloroquinolin-4-yl)-N^2-(3-(trifluoromethyl)benzyl)benzoxazole-2,5-diamine,* **32**

43 % yield. mp 108-110 °C. ^{1}H NMR (400 MHz, CD_3OD) δ 8.31 (d, *J*=5.6 Hz, 1H), 8.26 (d, *J*=9.0 Hz, 1H), 7.84 (d, *J*=2.2 Hz, 1H), 7.73 – 7.70 (m, 1H), 7.69 – 7.65 (m, 1H), 7.60 – 7.51 (m, 2H), 7.46 (dd, *J*=9.0, 2.2 Hz, 1H), 7.34 (d, *J*=8.4 Hz, 1H), 7.24 (d, *J*=2.1 Hz, 1H), 7.04 (dd, *J*=8.4, 2.1 Hz, 1H), 6.75 (d, *J*=5.6 Hz, 1H), 4.67 (s, 2H). ^{13}C NMR (101 MHz, CD_3OD) δ 163.62, 150.99, 150.75, 148.75, 146.22, 143.50, 139.81, 136.00, 135.30, 130.69, 129.06, 126.35, 125.17, 123.89, 123.85, 123.81, 123.63, 123.59, 123.21, 117.89, 117.59, 111.90, 108.95, 100.84, 45.41. Anal. RP-HPLC t_R = 14.84 min (method A, purity >99%). LRMS (EI+): *m/z* = 468.1 (exact mass for $C_{24}H_{16}ClF_3N_4O$ = 468.0965).

4.1.2.13. *N^5-(7-Chloroquinolin-4-yl)-N^2-(pyridin-2-ylmethyl)benzoxazole-2,5-diamine,* **33**

61 % yield. mp 118-120 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.57 (d, *J*=4.7 Hz, 1H), 8.41 (d, *J*=5.5 Hz, 1H), 7.99 (d, *J*=2.0 Hz, 1H), 7.97 (d, *J*=9.0 Hz, 1H), 7.69 (td, *J*=7.8 (×2), 1.8 Hz, 1H), 7.40 (dd, *J*=9.0, 2.0 Hz, 1H), 7.33 (d, *J*=7.8 Hz, 1H), 7.29 (d, *J*=2.0 Hz, 1H), 7.20 – 7.26 (m, 2H), 6.95 (dd, *J*=8.4, 2.0 Hz, 1H), 6.74 (d, *J*=5.5 Hz, 1H), 4.78 (s, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 162.96, 155.51, 151.22, 149.33, 149.14, 149.01, 146.84, 144.63, 136.79, 135.50, 135.38, 128.40, 125.96, 122.62, 121.76, 121.40, 117.66, 117.37, 112.80, 109.34, 101.89, 47.47. Anal. RP-HPLC t_R = 11.29 min (method A, purity >99%). LRMS (EI+): *m/z* = 400.9 (exact mass for $C_{22}H_{16}ClN_5O$ = 401.1043).

4.1.2.14. *N^5-(7-Chloroquinolin-4-yl)-N^2-(2-(pyridin-2-yl)ethyl)benzoxazole-2,5-diamine,* **34**

67 % yield. mp 89-91 °C. ^{1}H NMR (400 MHz, DMSO-*d6*) δ 9.01 (s, 1H), 8.52 – 8.47 (m, 1H), 8.42 (d, *J*=9.0 Hz, 1H), 8.38 (d, *J*=5.4 Hz, 1H), 8.06 (t, *J*=5.7 Hz, 1H), 7.85 (d, *J*=2.2 Hz, 1H), 7.69 (td, *J*=7.8 (×2), 1.9 Hz, 1H), 7.52 (dd, *J*=9.0, 2.2 Hz, 1H), 7.36 (d, *J*=8.4 Hz, 1H), 7.29 (d, *J*=7.8 Hz, 1H), 7.24 – 7.17 (m, 2H), 6.93 (dd, *J*=8.4, 2.1 Hz, 1H), 6.70 (d, *J*=5.4 Hz, 1H), 3.74 –

3.63 (m, 2H), 3.07 (t, *J*=7.2 Hz, 2H). ^{13}C NMR (101 MHz, DMSO-*d6*) δ 163.51, 159.25, 152.31, 149.90, 149.81, 149.57, 145.97, 144.88, 136.94, 136.21, 134.28, 128.01, 125.15, 124.85, 123.76, 122.04, 118.41, 116.89, 112.08, 109.31, 101.54, 42.53, 37.56. Anal. RP-HPLC t_R = 13.20 min (method A, purity >99%). LRMS (EI+): *m/z* = 415.1 (exact mass for $C_{23}H_{18}ClN_5O$ = 415.1200).

4.1.2.15. ***N*^{5}*-(7-Chloroquinolin-4-yl)-N*^{2}*-(pyridin-3-ylmethyl)benzo[d]oxazole-2,5-diamine,* 35**

58 % yield. mp 108-109 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.66 (d, *J*=2.2 Hz, 1H), 8.54 (dd, *J*=4.8, 1.6 Hz, 1H), 8.46 (d, *J*=5.4 Hz, 1H), 8.00 (d, *J*=2.1 Hz, 1H), 7.90 (d, *J*=9.0 Hz, 1H), 7.78 – 7.71 (m, 1H), 7.42 (dd, *J*=9.0, 2.2 Hz, 1H), 7.30 – 7.25 (m, 2H), 7.24 (d, *J*=2.2 Hz, 1H), 6.96 (dd, *J*=8.4, 2.2 Hz, 1H), 6.76 (d, *J*=5.4 Hz, 1H), 4.70 (s, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 162.66, 151.84, 149.66, 149.40, 149.19, 148.79, 146.63, 144.29, 135.79, 135.26, 133.17, 129.01, 125.92, 123.58, 121.07, 117.79, 117.58, 112.90, 109.47, 102.08, 77.25, 76.93, 76.61, 44.76. Anal. RP-HPLC t_R = 10.87 min (method A, purity >99%). LRMS (EI+): *m/z* = 401.1 (exact mass for $C_{22}H_{16}ClN_5O$ = 401.1043).

4.1.2.16. ***N*^{5}*-(7-Chloroquinolin-4-yl)-N*^{2}*-(pyridin-4-ylmethyl)benzoxazole-2,5-diamine,* 36**

23 % yield. mp 202-204 °C. ^{1}H NMR (300 MHz, CD_3OD) δ 8.52 – 8.43 (m, 2H), 8.31 (d, *J*=5.6 Hz, 1H), 8.27 (d, *J*=9.0 Hz, 1H), 7.84 (d, *J*=2.1 Hz, 1H), 7.51 – 7.42 (m, 1H, 3H), 7.36 (d, *J*=8.4 Hz, 1H), 7.23 (d, *J*=2.1 Hz, 1H), 7.05 (dd, *J*=8.4, 2.1 Hz, 1H), 6.75 (d, *J*=5.6 Hz, 1H), 4.67 (s, 2H). ^{13}C NMR (101 MHz, CD_3OD) δ 163.58, 150.96, 150.78, 149.12, 148.86, 148.72, 146.29, 143.44, 136.05, 135.34, 126.33, 125.20, 123.22, 122.26, 117.89, 117.72, 111.99, 109.03, 100.83, 44.73. Anal. RP-HPLC t_R = 10.06 min (method B, purity 96%). LRMS (EI+): *m/z* = 401.1 (exact mass for $C_{22}H_{16}ClN_5O$ = 401.1043).

4.1.2.17. ***N*^{5}*-(7-Chloroquinolin-4-yl)-N*^{2}*-(4-methylpiperazin-1-yl)benzoxazole-2,5-diamine,* 37**

49 % yield. mp 80-81 °C. ^{1}H NMR (400 MHz, CD_3OD) δ 8.29 (d, *J*=5.6 Hz, 1H), 8.25 (d, *J*=9.0 Hz, 1H), 7.82 (d, *J*=2.1 Hz, 1H), 7.44 (dd, *J*=9.0, 2.1 Hz, 1H), 7.34 (d, *J*=8.5 Hz, 1H), 7.25 (d, *J*=2.1 Hz, 1H), 7.03 (dd, *J*=8.5, 2.1 Hz, 1H), 6.74 (d, *J*=5.6 Hz, 1H), 3.75 – 3.64 (m, 4H), 2.60 – 2.51 (m, 4H), 2.33 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 163.15, 151.79, 149.49, 149.08, 146.81, 145.15, 135.31, 135.17, 128.76, 127.75, 125.76, 121.39, 116.30, 112.03, 109.01, 101.85, 43.05

(×2), 13.46 (×2). Anal. RP-HPLC t_R = 8.87 min (method A, purity >99%). LRMS (EI+): *m/z* = 408.1 (exact mass for $C_{21}H_{21}ClN_6O$ = 408.1465).

4.1.2.18. *N^5-(7-Chloroquinolin-4-yl)-N^2,N^2-diethylbenzoxazole-2,5-diamine,* 38

58 % yield. mp 189-190 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.45 (d, *J*=5.4 Hz, 1H), 7.99 (d, *J*=2.2 Hz, 1H), 7.90 (d, *J*=8.9 Hz, 1H), 7.39 (dd, *J*=8.9, 2.2 Hz, 1H), 7.23 – 7.25 (m, 2H), 6.88 (dd, *J*=8.3, 2.2 Hz, 1H), 6.76 (d, *J*=5.4 Hz, 1H), 3.59 (q, *J*=7.2 Hz, 4H), 1.29 (t, *J*=7.2 Hz, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 163.15, 151.79, 151.67, 149.49, 149.08, 146.81, 145.15, 135.31, 135.17, 128.76, 125.76, 121.39, 116.30, 112.03, 109.01, 101.85, 43.05 (×2), 13.46 (×2). Anal. RP-HPLC t_R = 12.97 min (method A, purity >99%). LRMS (EI+): *m/z* = 366.1 (exact mass for $C_{20}H_{19}ClN_4O$ = 366.1247).

4.1.2.19. *N-(7-Chloroquinolin-4-yl)-2-(4-methylpiperazin-1-yl)benzoxazol-5-amine,* 39

47 % yield. mp 113-115 °C. ^{1}H NMR (400 MHz, DMSO-*d6*) δ 9.02 (s, 1H), 8.41 (d, *J*=9.0 Hz, 1H), 8.38 (d, *J*=5.4 Hz, 1H), 7.85 (d, *J*=2.2 Hz, 1H), 7.52 (dd, *J*=9.0, 2.2 Hz, 1H), 7.43 (d, *J*=8.2 Hz, 1H), 7.23 (d, *J*=2.0 Hz, 1H), 6.98 (dd, *J*=8.2, 2.0 Hz, 1H), 6.70 (d, *J*=5.4 Hz, 1H), 3.60 (t, *J*=5.0 Hz, 4H), 2.42 (t, *J*=5.0 Hz, 4H), 2.22 (s, 3H). ^{13}C NMR (101 MHz, DMSO-*d6*) δ 163.04, 152.38, 150.03, 149.68, 146.20, 144.55, 136.64, 134.27, 128.10, 125.15, 124.82, 118.49, 117.21, 112.20, 109.72, 101.64, 54.18, 46.21, 45.66. Anal. RP-HPLC t_R = 11.50 min (method A, purity >99%). LRMS (EI+): *m/z* = 393.1 (exact mass for $C_{21}H_{20}ClN_5O$ = 393.1356).

4.1.2.20. *N-(7-Chloroquinolin-4-yl)-2-(4-phenylpiperazin-1-yl)benzoxazol-5-amine,* 40

74 % yield. mp 96-98 °C. ^{1}H NMR (400 MHz, DMSO-*d6*) δ 9.03 (s, 1H), 8.42 (d, *J*=9.0 Hz, 1H), 8.39 (d, *J*=5.3 Hz, 1H), 7.86 (d, *J*=2.2 Hz, 1H), 7.52 (dd, *J*=9.0, 2.2 Hz, 1H), 7.45 (d, *J*=8.4 Hz, 1H), 7.29 – 7.16 (m, 3H), 7.05 – 6.94 (m, 3H), 6.82 (t, *J*=7.2 Hz, 1H), 6.72 (d, *J*=5.3 Hz, 1H), 3.75 (t, *J*=5.0 Hz, 4H), 3.27 (t, *J*=5.0 Hz, 4H). ^{13}C NMR (101 MHz, DMSO-*d6*) δ 163.07, 152.34, 151.29, 150.17, 149.80, 146.38, 144.64, 136.96, 134.29, 129.46, 128.17, 125.08, 124.80, 119.98, 118.68, 117.29, 116.60, 112.33, 109.69, 101.94, 48.52, 45.73. Anal. RP-HPLC t_R = 15.41 min (method A, purity >99%). LRMS (EI+): *m/z* = 455.1 (exact mass for $C_{26}H_{22}ClN_5O$ = 455.1513).

4.1.2.21. *2-(4-(3-Chlorophenyl)piperazin-1-yl)-N-(7-chloroquinolin-4-yl)benzoxazol-5-amine,* **41**

40 % yield. mp 90-92 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.46 (d, *J*=5.3 Hz, 1H), 8.00 (d, *J*=2.1 Hz, 1H), 7.89 (d, *J*=9.0 Hz, 1H), 7.40 (dd, *J*=9.0, 2.1 Hz, 1H), 7.31 – 7.25 (m, 2H), 7.24 – 7.15 (m, 1H), 6.95 (dd, *J*=8.4, 2.1 Hz, 1H), 6.92 (t, *J*=2.2 Hz, 1H), 6.89 – 6.86 (m, 1H), 6.84 – 6.80 (m, 1H), 6.77 (d, *J*=5.4 Hz, 1H), 3.92 – 3.80 (m, 4H), 3.38 – 3.22 (m, 4H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 162.89, 152.03, 151.82, 149.68, 148.89, 146.77, 144.54, 135.89, 135.21, 130.16, 128.93, 125.82, 121.22, 120.45, 117.86, 117.19, 116.73, 114.69, 112.61, 109.36, 102.07, 48.67, 45.47. Anal. RP-HPLC t_R = 15.94 min (method A, purity >99%). LRMS (EI+): *m/z* = 489.1 (exact mass for $C_{26}H_{21}Cl_2N_5O$ = 489.1123).

4.1.2.22. *N-(7-Chloroquinolin-4-yl)-2-((dimethylamino)methyl)benzoxazol-5-amine,* **42**

49 % yield. mp 156-158 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.37 (d, *J* = 5.4 Hz, 1H), 7.88 – 7.94 (m, 1H, 1H), 7.54 (d, *J* = 2.1 Hz, 1H), 7.46 (d, *J* = 8.6 Hz, 1H), 7.30 (dd, *J* = 9.0, 2.2 Hz, 1H), 7.21 (dd, *J* = 8.6, 2.1 Hz, 1H), 6.68 (d, *J* = 5.4 Hz, 1H), 3.73 (s, 2H), 2.34 (s, 6H). ^{13}C NMR (101 MHz, CD_3OD) δ 164.75, 151.11, 150.36, 148.82, 148.48, 141.53, 136.86, 135.39, 126.47, 125.36, 123.21, 122.28, 118.03, 115.03, 111.18, 100.96, 55.09, 44.04. Anal. RP-HPLC t_R = 2.78 min (method C, purity 98%). LRMS (EI+): *m/z* = 352.0 (exact mass for $C_{19}H_{17}ClN_4O$ = 352.1091).

4.1.2.23. *N-(7-Chloroquinolin-4-yl)-2-(2-methoxyethyl)benzoxazol-5-amine,* **43**

65 % yield. mp 165-166 °C. ^{1}H NMR (300 MHz, CD_3OD) δ 8.33 (d, *J*=5.5 Hz, 1H), 8.27 (d, *J*=9.0 Hz, 1H), 7.84 (d, *J*=2.1 Hz, 1H), 7.63 – 7.59 (m, 2H), 7.47 (dd, *J*=9.0, 2.2 Hz, 1H), 7.35 (dd, *J*=8.6, 2.2 Hz, 1H), 6.77 (d, *J*=5.5 Hz, 1H), 3.89 (t, *J*=6.2 Hz, 2H), 3.37 (s, 3H), 3.21 (t, *J*=6.2 Hz, 2H). ^{13}C NMR (101 MHz, CD_3OD) δ 167.58, 156.58, 150.21, 142.90, 142.26, 140.17, 139.21, 133.37, 127.89, 124.91, 122.88, 119.14, 116.70, 115.91, 111.71, 100.24, 68.50, 57.54, 28.89. Anal. RP-HPLC t_R = 12.83 min (method A, purity >99%). LRMS (EI+): *m/z* = 353.1 (exact mass for $C_{19}H_{16}ClN_3O_2$ = 353.0931).

4.2. In vitro *P. falciparum* assay and in vivo antimalarial efficacy studies.

Compounds were screened against multidrug resistant (K1) and sensitive (NF54) strains of *P. falciparum* in vitro using the modified [^{3}H]-hypoxanthine incorporation assay.[36] In vivo efficacy was conducted as previously described,[37] with the modification that mice (n = 3) were infected with a GFP-transfected *P. berghei* ANKA strain (donated by A. P. Waters and C. J. Janse, Leiden University, The Netherlands), and parasitemia was determined using standard flow cytometry techniques. The detection limit was 1 parasite in 1,000 erythrocytes (that is, 0.1%). Activity was calculated as the difference between the mean per cent parasitaemia for the control and treated groups expressed as a per cent relative to the control group. Compounds were dissolved or suspended in 70/30 Tween 80/ethanol, diluted 10× with water and orally administered once per day on four consecutive days (4, 24, 48 and 72 h after infection). Blood samples for the quadruple-dose regimens were collected on day 4 (96 h after infection).

4.3. β-Hematin formation inhibition assay

The β-hematin formation inhibition assay method described by Carter et al. was modified for manual liquid delivery [38,39]. Two stock solutions of the samples were prepared by dissolving the pre-weighed compound in DMSO with sonication to give 20 mM and 2 mM solutions of each sample. These were delivered to a 96-well plate in duplicate to give concentrations ranging from 0–1000 μM (final well concentration) with a total DMSO volume of 10 μL in each well, after which deionised 1HO (70 μL) and NP-40 (20 μL; 30.55 μM) were added. Plates containing coloured compounds were pre-read on a on a SpectraMax plate reader for blanking purposes. A 25 mM haematin stock solution was prepared by sonicating hemin in DMSO for one minute and then suspending 178 μL of this in 1M acetate buffer (pH 4.8). The homogenous suspension (100

μL) was then added to the wells to give final buffer and hematin concentrations of 0.5 M and 100 μM respectively. The plate was covered and incubated at 37°C for 5–6 hrs. Free heme was detected using the pyridine-ferrichrome method developed by Ncokazi and Egan [40]. A solution of 50 % (v/v) pyridine, 30 % (v/v) 1HO, 20 % (v/v) acetone and 0.2 M HEPES buffer (pH 7.4) was prepared and 32 μL added to each well to give a final pyridine concentration of ~5 % (v/v). Acetone (60 μL) was then added to assist with haematin dispersion. The UV-vis absorbance of the plate wells was read on a SpectraMax® 340 PC^{384} Absorbance Microplate Reader (Molecular Devices, Sunnyvale, CA, USA). Sigmoidal dose-response curves were fitted to the absorbance data using GraphPad Prism v3.02 to obtain a 50 % inhibitory concentration (IC_{50}) for each compound.

4.4. Cytotoxicity assay against CHO cells

Test samples were screened for *in vitro* cytotoxicity against a mammalian cell-line, Chinese Hamster Ovarian (CHO) using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT)-assay. The MTT-assay is used as a colorimetric assay for cellular growth and survival, and compares well with other available assays.[41,42] The tetrazolium salt MTT was used to measure all growth and chemosensitivity. Test samples were tested in triplicate on one occasion. The test samples were prepared to a 20 mg/ml stock solution in 100 % DMSO. Stock solutions were stored at -20ºC. Further dilutions were prepared in complete medium on the day of the experiment. Samples were tested as a suspension if not completely dissolved. Emetine was used as the reference drug in all experiments. The initial concentration of emetine was 100 μg/ml, which was serially diluted in complete medium with 10-fold dilutions to give 6 concentrations, the lowest being 0.001 μg/ml. The same dilution technique was applied to the all test samples. The highest concentration of solvent to which the cells were exposed to had no measurable effect on the cell viability (data

not shown). The 50% inhibitory concentration (IC_{50}) values were obtained from full dose-response curves, using a non-linear dose-response curve fitting analysis via GraphPad Prism v.4 software.

4.5. Microsomal stability assay

This assay was conducted in 96-well plate format according to a previously reported method.[34] Test compounds and controls were prepared from 10 mM DMSO stock solutions. 0.40 mg protein/ml microsomes (pooled Human mixed gender, male Mouse BALB/c) from XenoTech were incubated with 1 mM test compound at 37 °C. Metabolic reactions were initiated by the addition of the co-factor NADPH and the plates were incubated for 30 minutes. The reactions were quenched with triple the volume of acetonitrile containing carbamazepine as internal standard. The centrifuged and filtered samples were analyzed by HPLC-MS/MS using either a Micromass single quadrupole, triple quadrupole or TOF mass spectrometer (Waters Corporation, Milford, MA) to determine the remaining concentrations of the test compounds. Control standards (midazolam and propranolol) were included in the assay to provide quality control and an indication of the metabolic capacity of the microsomes used.

4.6. Pharmacokinetic evaluation in mice

In vivo pharmacokinetics was analysed in six, 8-week-old male C57BL/6 mice divided into an intravenous and oral dosage group, n = 3. After a single 5 mg/kg intravenous injection of **24** into the penile dorsal vein (formulation; DMSO, PEG, EtOH, PPG (2:6:1:7, v/v)), 20 µl of blood was collected from the tail tip at 0.08, 0.5, 1, 3, 7 and 24 h and stored at - 80°C. The oral groups received a single 20 mg/kg dose by oral gavage of the selected compound suspended in a 0.5% HPMC in water solution and blood collected was at 0.5, 1, 3, 5 and 24 h. Whole blood concentration of the

compounds were quantified by an LC-MS/MS assay developed for a range of 10 – 5000 ng/ml. The samples were extracted by protein precipitation using 20 µl whole blood and 80 µl methanol. Gradient chromatography was performed on a Waters Xterra™ MS C18 (2.1 × 30mm, 3.5 µm) reverse phase column with mobile phase 0.1% ammonium hydroxide:water (v/v) and 0.1% ammonium hydroxide:acetonitrile (v/v) at a flow rate of 400 µl/min. An AB Sciex API 3200 mass spectrometer was operated at unit resolution in multiple reaction monitoring mode, monitoring the transitions of the protonated molecular ions to its product ion of 436.3 → 351.2. The accuracies (%Nom) were between 88.1% and 109.3% at lowest level of quantification (10 ng/ml), medium (2000 ng/ml) and high (4000 ng/ml) quality controls. Non-compartmental analysis was performed on **24** using Summit PK solutions™ (Summit Research Services, Montrose, USA).

SUPPLEMENTARY CONTENT:

Supporting Information. HPLC conditions for purity checks, ^{1}H-NMR spectra of selected compounds and additional compound data.

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ABBREVIATIONS USED: p.o., oral administration; i.v., intraveneous administration; MSD, mean survival days; PK, pharmacokinetics; AUC, area under curve; TLC, thin layer chromatography; ADQ, amodiaquine; ACN, acetonitrile; mp, melting point; eq, equivalent.

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