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Antimalarial Pyrido[1,2-*a*]benzimidazole Derivatives with Mannich Base Side Chains: Synthesis, Pharmacological Evaluation and Reactive Metabolite Trapping Studies

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A novel series of pyrido[1,2-*a*]benzimidazoles bearing Mannich base side chain and their metabolites were synthesised and evaluated for antiplasmodium activity, microsomal metabolic stability, reactive metabolite (RM) formation and in vivo antimalarial efficacy in a mouse model. Oral administration of one of the derivatives at 4x50 mg/kg reduced parasitemia by 95% in *Plasmodium berghei*-infected mice, with a mean survival period of 16 days post treatment. The in vivo efficacy of these derivatives is likely a consequence of their active metabolites, two of which showed potent antiplasmodium activity against chloroquine-sensitive and multidrug resistant *P. falciparum* strains. Rapid metabolism was observed for all the analogues with < 40% of parent compound remaining after 30 minutes of incubation in liver microsomes. RM trapping studies detected glutathione adducts only in derivatives bearing 4-hydroxyl, with fragmentation signatures showing that this conjugation occurred on the phenyl ring of the Mannich base side chain. As with amodiaquine (AQ), interchanging the positions of the 4-hydroxyl and Mannich base side group or substituting the 4-hydroxyl with fluorine appeared to block bioactivation of the AQ-like derivatives though at the expense of antiplasmodial activity, which was significantly lowered.

KEYWORDS: *Plasmodium falciparum*, Pyrido[1,2-*a*]benzimidazoles, Mannich base, Amodiaquine, 4-aminophenol, Reactive metabolite

Malaria constitutes one of the most important human parasitic diseases due to its impact on public health and economies, especially in low-income countries.[1] The World Health Organization (WHO) estimates that there were ~216 million malaria cases and ~445 000 related deaths globally in 2016.[2] Though these statistics attest to successful global expansion in control efforts in the last 15 years, they still remain troublingly high and highlight the numerous impediments to mitigation strategies, which include reduced sensitivity to the WHO-recommended first-line artemisinin combination therapy (ACT)[3] and development of insecticide resistance in malaria vectors.[4] Furthermore, the only licensed vaccine (RTS,S) is plagued by limited efficacy,[5] lacks pan-protection and is currently introduced only as a pilot scheme in a few settings in sub-Saharan Africa. These underscore the urgent need for novel alternative therapeutic options.

In an effort to explore new chemotypes for antimalarial drug development, we recently reported on the efficacy of pyrido[1,2-*a*]benzimidazoles (PBIs),[6, 7] which have previously been investigated for their antitumor and antimicrobial activity.[8] Interestingly, PBIs share an architectural semblance with the 4-aminoquinoline antimalarials chloroquine (CQ) (**A**) and amodiaquine (AQ) (**B**) (**Figure 1**), including an electron-dense planar heterocyclic moiety, a basic amine side group and a putative mode of action involving inhibition of the haem detoxification pathway,[7, 9] presumably due to their ability to adopt a flat conformation. Although AQ (in fixed dose combination with artesunate) is now an integral constituent of ACT, its previous use as monotherapy for prophylaxis was associated with agranulocytosis and life-threatening hepatotoxicity[10] attributed to its ability to undergo oxidative metabolism to the chemically reactive quinoneimine (**C**) and aldehyde quinoneimine (**D**) metabolites.[11, 12] Within cells, this reactive metabolite (RM) is trapped by the antioxidant glutathione (GSH) and its precursor *N*-acetylcysteine, and the resultant conjugates have been detected in granulocytes as well as in the bile of experimental animals.[13, 14] Upon depletion of GSH, the

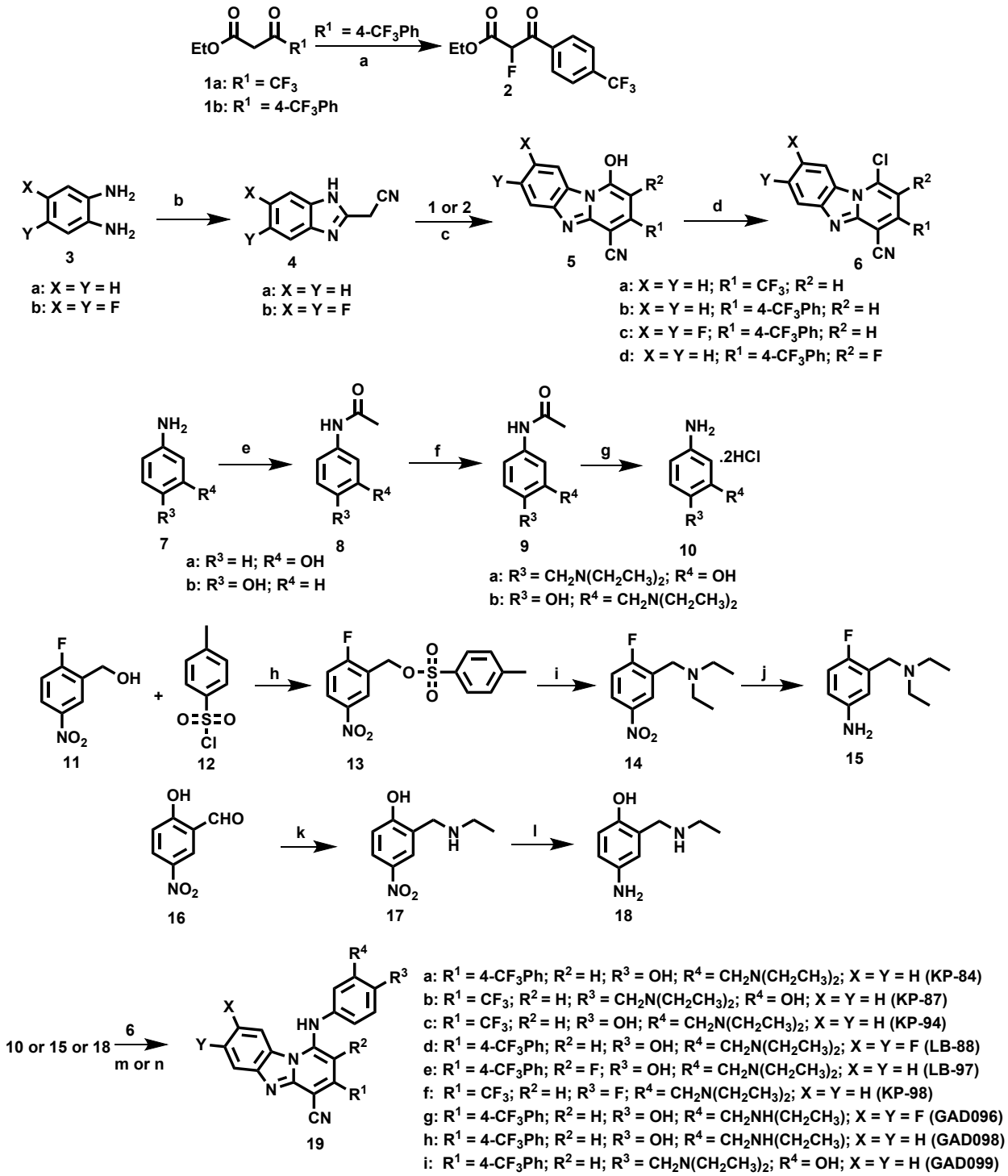
quinoneimine reacts with other soft nucleophiles in the cell leading to toxicity. Crucially, the formation of this RM species requires the presence of a 4-aminophenol substructure that permits formation of the electrophilic quinoneimine. A number of antimalarial agents in development and clinical use, like pyronaridine (**E**), amopyroquine (**F**), tebuquine (**G**) and cycloquine (**H**) (**Figure 1**), coincidentally contain this 4-aminophenol motif, which is likely to impact their toxicological profiles. Additionally, incorporation of an amine group in their side chains further classes some of these AQ-like molecules as Mannich base antimalarials.

To bypass cellular toxicity from the facile oxidation of AQ, a number of strategies have been reported. For example, replacement of the 4'-hydroxyl group with 4'-fluorine atom yielded 4'-dehydroxy-4'-fluoroamodiaquine (**I**) which was shown to exhibit potent antiplasmodium activity.[14, 15] Miroshnikova and colleagues also synthesized a series of isotebuquine analogues (**J**) with good antimalarial activity albeit blemished by poor oral bioavailability.[16] More famously, O'Neill and colleagues devised an approach involving the interchanging of the 3-hydroxyl and 4-Mannich side-chain of AQ to afford new analogues lacking the potential to form RMs via this quinoneimine pathway, while retaining potent antimalarial activity.[17] Among these derivatives, isoquine (**K**) proved efficacious in animal models and showed no evidence of in vivo bioactivation.[17] Further development of **K** was, however, compromised by unacceptably high first pass metabolism to de-alkylated metabolites detected during preclinical evaluation leading to diminished activity against CQ-resistant (CQR) parasite strains.[18] The metabolically less labile *N*-*tert*-butyl isoquine (**L**) (**Figure 2**) was subsequently developed but, despite its comparatively superior pharmacokinetic and pharmacodynamic profiles, inability to achieve exposures at doses considered to demonstrate superior safety compared with CQ led to its discontinuation. As back up candidate for **L**, 4'-fluoro-*N*-*tert*-butylamodiaquine (**M**) (**Figure 2**) was developed with a fluorine replacement of the 3'-hydroxyl and 4'-*N*- *tert*-butyl instead of the 3'-*N*-diethyl to block the *N*-dealkylation

site. This molecule, with a suitable oral bioavailability and acceptable safety profile underwent preclinical evaluation.[19] Even more recently, our research group also reported a novel approach to block the formation of both the quinoneimine and aldehyde metabolites based on the hypothesis that AQ analogues in which the hydroxyl group and the Mannich base side chains are cyclized into a benzothiazole (**N**), benzoxazole (**O**) or benzimidazole (**P**) ring system will not form RMs.[20, 21]

Pursuant to the abovementioned strategies, this article presents data on the synthesis, antiplasmodium activity as well as microsomal metabolic profiles of a series of PBI derivatives bearing Mannich base side chains and/or designed to ablate their RM-forming ability. These comprise analogues with the hydroxyl and Mannich side group either interchanged at the C3 or C4 positions or the hydroxyl substituted with fluorine on the background of various substitutions at C2, C3 and the left hand side (LHS) of the PBI nucleus (**Figure 3**). As proof-of-concept, we also synthesised and tested the *N*-deethylation metabolites of some of the derivatives to highlight their potency. To gain insight into the ability of this series to undergo bioactivation and the compounds' antimalarial efficacy, a representative set of analogues was tested for RM formation and in vivo activity in a murine malaria model.

Scheme 1: Synthesis of pyrido[1,2-*a*]benzimidazole (PBI) derivatives [a]



Scheme 1: Reagents and reaction conditions: (a) Selectfluor^{TM}, MeCN, microwave (150 W), 82 °C, 10 min. (b) Ethyl cyanoacetate, DMF, microwave, 110 °C, 15 min. (c) NH_4OAc, 150 °C, 1 h; (d) $POCl_3$, 130 °C, 2 h. (e) $(CH_3CO)_2O$, THF, 60 °C, 30 min. (f) HCHO, Et_3NH, EtOH, microwave, 80 °C, 1 h. (g) 6N HCl, 100 °C, 2 h. (h) aq. NaOH, THF, 5-10 °C, 2 h. (i)

Et_2NH, Et_3N, Dioxane, 60 °C, 4 h. (j) $SnCl_2$, 1N HCl, THF, 60 °C, 8 h. (k) $EtNH_2$, MeOH, 25 ^oC, 1 h/0 ^oC, $NaBH_4$, 16 h (l) H_2, Pd/C, MeOH, 25 ^oC, 16 h. (m) Et_3N, THF:DMF, microwave, 80 °C, 20 min. (n) EtOH, HCl, microwave, 80 °C, 1.30 h (for **19g-h**).

RESULTS AND DISCUSSION

***Chemistry*:** The synthesis of target PBI derivatives **19a-i** was relatively straightforward using published procedures (**Scheme 1**).[7, 15, 17] Briefly, ethyl acetoacetate **1** (R^1 = 4-CF_3Ph) was treated with Selectfluor™ to afford starting material ethyl 2-fluoro-3-oxo-3-(4-(trifluoromethyl)phenyl)propanoate, **2.** Acetonitrile **4b** was synthesized by reacting commercially available 1,2-diaminobenzene **3b** with ethylcyanoacetate in DMF under microwave irradiation. Subsequent reaction of commercially available acetonitrile **4a** and **4b** with ethyl acetoacetates **1a/2** in the presence of ammonium acetate afforded the tricyclic intermediates **5,** which were then converted to the key chloro intermediates **6a-d** on reaction with $POCl_3$. The Mannich bases **10a-b** were synthesized as previously described.[15, 17] The flourinated Mannich base **15** was synthesized by activating the hydroxyl group of commercially available **11** with *p*-toluenesulfonylchloride to afford **13** followed by reaction with diethylamine and subsequent nitro group reduction. Reductive amination of commercially available 2-hydroxy-5-nitrobenzaldehyde **16** with ethylamine to yield **17**, followed by reduction of the nitro group afforded **18**, the *N*-desethyl analogue of **10b**. The obtained Mannich bases described above were then reacted with the chloro intermediates **6a-d** to afford the desired PBI derivatives **19a-i**.

***Cytotoxicity and In Vitro Antiplasmodium Activity*:** All compounds were evaluated for in vitro antiplasmodium activity against the CQ-sensitive (*Pf*NF54) and the multidrug resistant (*Pf*K1) strains as well as mammalian cytotoxicity against a Chinese hamster ovary (CHO) cell line. *Pf*NF54 and *Pf*K1 were chosen as controls to offer insight into the full spectrum of compound activity against *P. falciparum* as they broadly represent drug-sensitive and –resistant strains, respectively. The results are summarized in **Table 1**. In summary, these compounds showed a favourable cytotoxicity profile with an S.I range between 16 and 1718 (**Table 1**). Emetine, a potent alkaloid inhibitor of eukaryotic protein synthesis, was used as control and, expectedly, exhibited cellular toxicity at extremely low concentrations.

Antiplasmodium activity ranged from 0.07 – 5.14 μM and 0.07 - 6.62 μM against *Pf*NF54 and *Pf*K1, respectively, with 4/9 compounds exhibiting submicromolar potency against both strains. Introduction of fluorine atoms at C7 and C8 positions on the LHS of the PBI core while maintaining the para-trifluoromethyl phenyl (4-CF_3Ph) substitution at the C3 on the PBI core appeared to afford the most active compounds (**19d** and **19g**) with an equally good cytotoxicity profile, thus confirming previous observation of significant improvement in activity upon mono- or di- halo-substitution on the LHS.[7] On the other hand, replacing the para-trifluoromethyl phenyl in **19a** with CF_3 at C3 (as in **19c**) led to an eight-fold drop in potency albeit with retention of some activity. Nevertheless, the same replacement while interchanging the positions of the hydroxyl and Mannich base side group (**19b**) or substituting the 4-hydroxyl with fluorine (**19f**) was significantly detrimental to activity.

***Microsomal Metabolic Stability and Metabolite Identification Studies*:** Microsomal metabolic stability was investigated using a single point metabolic stability assay involving incubation at 37 °C with human and/or mouse liver microsomes in the presence of phosphate

buffer and NADPH as co-factor.[22] All compounds were rapidly metabolized, with < 40% of the parent compounds remaining after 30 minutes of incubation in microsomes (**Table 2**). This was characterized by short degradation half-lives, suggesting significant susceptibility to hepatic metabolism regardless of the modification on the aromatic ring of the Mannich side group. Di-substitution with fluorine at C7 and C8 positions of the LHS (**19d**) did not improve metabolic stability. No metabolism occurred in the absence of NADPH or microsomes, implying that the observed biotransformation was primarily a function of cytochrome p450 enzyme-mediated phase 1 metabolism with *N*-deethylation seemingly the predominant route of metabolism.

***Bioactivation Experiments*:** To investigate RM formation, five compounds comprising those in which the 4-aminophenol substructure is maintained (**19a**, **19c** and **19d**) and others bearing modification on this substructure (**19b** and **19f**) were screened by LC-MS/MS in rat liver microsomes. Using GSH as a trapping agent, GSH-drug adducts were detected only in samples incubated with **19a**, **19c** and **19d** (**Figure 4**). Analysis of the fragmentation patterns of the GSH conjugates in positive electrospray ionisation mode revealed $[M+H-73]^+$ ions, corresponding to the cleavage of the diethylamino group. Subsequent neutral loss of 129 Da was characteristic of a GSH conjugate, while a neutral loss of 273 Da indicated that the GSH conjugation likely occurred on the phenyl ring of the Mannich base side chain[23] (Supporting Information **Figure S1** and **S2**, **Table S1**). Expectedly, modification of the structure to circumvent formation of the reactive quinoneimine appeared successful in **19b** (positions of the hydroxyl and Mannich side group interchanged) and **19f** (4'-fluoro replacement of the 4'-hydroxyl).

In vivo Antimalarial Efficacy: On the basis of good antiplasmodium activity and cytotoxicity profile, **19a** was chosen for proof-of-concept study - specifically the assessment of in vivo efficacy in *P. berghei*-infected mice at an oral dose of 4x50 mg/kg (**Table 3**). Although no mice were cured of infection (0/3), parasitemia was reduced by 95% and the animals survived slightly longer (MSD: 16) compared to **KP4** (MSD: 14), a prototype PBI molecule with previously confirmed in vivo efficacy.[7] Noteworthy, AQ itself is poorly stable in human liver microsomes (CL_{int} of 608 µL/min/mg), with a short elimination half-life of ~5 hours in humans[24] and its pharmacological activity is dominantly sustained by its stable longer-circulating *N*-deethylation metabolite, desethylamodiaquine.[25] Curiously, the main metabolites of all the derivatives were analogous to the active metabolite of AQ, possibly explaining the significant reduction in parasitemia observed for **19a**, regardless of the anticipated high in vivo metabolism. We accordingly synthesized and evaluated **19h**, the *N*-deethylation metabolite of **19a**. It is likely that this metabolite persists in systemic circulation long enough to suppress parasitemia even after the concentration of the parent compound has abated below the therapeutic threshold. Indeed, the potent in vitro antiplasmodium activity data of **19h** (**Table 1**) and the extended elimination half-life of desethylamodiaquine (6 - 18 days) support this assertion. Compound **19c**, tested alongside **19a**, only elicited <40% reduction in parasitemia in the mice, and we speculate that this could be due to poor oral bioavailability possibly occasioned by any one or more of its physicochemical attributes compounded by its low metabolic stability.

In conclusion, modifications around the reactive quinoneimines of PBI derivatives with Mannich base side chains led to identification of compounds with good antiplasmodium activity, with retention of the para-trifluoromethyl phenyl group at the C3 position of the PBI core critical to biological activity. Strategies to circumvent bioactivation were successful, though at the expense of antiplasmodium activity. Amongst the most active compounds, **19a**

showed good in vivo activity in the mouse *P. berghei* model. Biotransformation data suggest that the in vivo activity of these compounds could be a consequence of their metabolites, two of which were in fact endowed with potent antiplasmodium activity against both CQS and multidrug resistant parasite strains. These metabolites can form the basis of a future lead optimization effort.

Experimental Procedures: ***General Method*****:** 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. ^{13}C NMR spectra were recorded at 75 MHz on a Varian Mercury Spectrometer or at 100 MHz on Varian Unity Spectrometer. High-resolution mass spectra were recorded on a VG70 SEQ micromass spectrometer. Melting points (m.p.) were determined by Differential Scanning Calorimetry (DSC) using TA Q200/Q2000 DSCfrom TA Instruments. 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). Chemical shifts (δ) are given in ppm downfield from TMS as the internal standard. Coupling constants, *J*, are recorded in Hertz (Hz). Purity was determined by HPLC and all compounds were confirmed to have > 95% purity. Any data that is not shown below is supplied in the **Supporting Information**.

Ethyl 2-fluoro-3-oxo-3-(4-(trifluoromethyl)phenyl)propanoate **2**:

Ethyl(4-trifluromethylbenzoyl)acetate **1b** (1.0 equiv.) was added to Selectfluor fluorinating agent (2.0 equiv.) in acetonitrile (3.0 mL). The mixture was heated in the microwave at 82°C for 10 minutes. The black precipitate was filtered off and the solvent was removed *in vacuo*.

The residue was purified by flash column chromatography (EtOAc-Hex) to acquire compound as a yellow oil (66%); Rf 0.33 (10 % EtOAc-Hex); ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.17 (d, *J* = 7.6, 2H), 7.79 (d, *J* = 8.2, 2H), 5.86 (d, *J* = 48.8 Hz, 1H), 4.33 (q, *J* = 7.1 Hz, 2H), 1.29 (t, *J* = 7.1 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 188.8, 164.3, 136.0, 129.9, 129.8, 125.8, 125.8, 121.9, 91.2, 89.3, 62.9 and 13.8; ^{19}F NMR (377 MHz, $CDCl_3$) δ -63.49, -190.44; MS: *m/z* 277.0 $[M-H]^-$.

2-(5,6-difluoro-1H-benzo[d]imidazol-2-yl)acetonitrile **4b**:

A mixture of 4,5-difluorobenzene-1,2-diamine **3b** (1.0 equiv.) and ethyl-2-cyanoacetate (2.0 equiv.) was heated in the microwave at 110°C for 15 minutes. The residue was purified by flash column chromatography (EtOAc-Hex) to obtain compound as a red/brown solid (60%); m.p. 267 - 269 °C; R_f 0.38 (70 % EtOAc-Hex); 1H NMR (400 MHz, DMSO) δ 12.80 (s, 1H), 7.67 – 7.59 (m, 2H), 4.39 (s, 2H); ^{13}C NMR (101 MHz, DMSO) δ 148.5, 148.3, 147.4, 146.1, 146.0, 116.8, 106.1, 100.6 and 18.9; MS: *m/z* 194.1 $[M+H]^+$.

General procedure for the synthesis of compound **5**:

A mixture of benzimidazole acetonitrile **4** (1.0 equiv.), NH_4OAc (2.0 equiv.), and the appropriate *β*-keto ester **1**/**2** (1.20 equiv.) was heated to reflux at 150 °C for 1 h and then allowed to cool to 100 °C. MeCN (10 mL) was added and the resulting mixture stirred for 15 min, allowed to cool to room temperature and then cooled on ice. The cold mixture was filtered and the residue washed with cold MeCN (4×10 mL), dried in vacuo, and used in the next step without further purification.

7,8-difluoro-1-hydroxy-3-(4-(trifluoromethyl)phenyl)benzo-[4,5]imidazo[1,2-*a*]pyridine-4-carbonitrile **5c**: Grey/brown solid (62 %); m.p. 289 - 294 °C; R_f 0.61 (70 % EtOAc-Hex); 1H NMR (400 MHz, DMSO) δ 8.52 (dd, *J* = 10.0, 7.0 Hz, 1H), 7.91 (d, *J* = 8.1 Hz, 2H), 7.84 (d,

J = 8.1 Hz, 2H), 7.63 (dd, *J* = 10.0, 7.0 Hz, 1H), 6.11 (s, 1H). ^{13}C NMR (101 MHz, DMSO) δ 158.1, 151.8, 149.1, 141.3, 130.5, 130.2, 129.6, 129.1, 129.0, 126.1, 126.1, 123.7, 123.6, 116.7, 105.7, 105.5, 105.1, 101.2 and 101.0; MS: *m/z* 388.0 $[M-H]^-$.

General procedure for the synthesis of compound **6:**

A mixture of compound **5** (1.0 equiv.) and $POCl_3$ (20 equiv.) was heated to reflux at 130 °C for 2 h. Excess $POCl_3$ was removed under reduced pressure and ice-cold water (20 mL) added to the residue, stirring to yield a precipitate. The mixture was neutralized with saturated $NaHCO_3$ and filtered. The resultant solid was washed with ice-cold water (4×15 mL), dried in vacuo, and used without further purification.

1-chloro-7,8-difluoro-3-(4-(trifluoromethyl)phenyl)benzo[4,5]imidazo[1,2-a]pyridine-4-carbonitrile **6c**: Brown solid (87 %); m.p. 237 - 242 °C; R_f 0.77 (70 % EtOAc-Hex); ^{1}H NMR (400 MHz, DMSO) δ 8.74 (dd, J = 11.0, 7.6 Hz, 1H), 8.13 (dd, J = 11.0, 7.6 Hz, 1H), 8.02 (s, 4H), 7.64 (s, 1H); ^{13}C NMR (150 MHz, DMSO) δ 148.5, 141.2, 141.1, 139.3, 134.8, 130.4 (2C), 126.4, 126.4, 125.8, 125.1, 125.0, 115.1, 113.3, 107.4, 107.2, 105.0, 104.8 and 97.6; MS: *m/z* 408.0 $[M+H]^+$.

General procedure for the synthesis of compound **8**

A mixture of corresponding aminophenol **7a-b** (1.0 equiv.) and acetic anhydride (1.0 equiv.) in THF was heated to reflux at 60 °C for 30 min. After completion of reaction (TLC), solvent was removed under reduced pressure and residue was washed with diethyl ether to obtained desired product **8**.

N-(3-Hydroxyphenyl)acetamide **8a:** Light pink solid (72%); R_f 0.4 (5% MeOH-DCM); m.p. 177-178°C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.74 (br s, 1H), 9.30 (br s, 1H), 7.18 (d, *J* = 2.1 Hz, 1H), 7.05 (t, *J* = 8.0 Hz, 1H), 6.92 (d, *J* = 8.1 Hz, 1H), 6.43 (ddd, *J* = 8.1, 2.4, 1.0 Hz, 1H), 2.02 (s, 3H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 168.6, 158.0, 140.8, 129.7, 110.5, 110.2, 106.7 and 24.5; MS: *m/z* 152 $[M+H]^+$.

N-(4-Hydroxyphenyl)acetamide **8b:** Light pink solid (78%); R_f 0.2 (5% MeOH-DCM); m.p. 166-167^{o}C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.61 (br s, 1H), 9.10 (br s, 1H), 7.35 (d, J = 8.9 Hz, 2H), 6.69 (d, J = 8.9 Hz, 2H), 1.99 (s, 3H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 167.9, 153.6, 131.5, 121.3 (2C), 115.4 (2C) and 24.1; MS: *m/z* 152 $[M+H]^+$.

General procedure for the synthesis of compound **9:**

Diethylamine (2.0 equiv.) and formaldehyde (2.0 equiv.) were added to a stirred mixture of compound **8a-b** (1.0 equiv.) in EtOH (5 mL) in microwave tube. The mixture was irradiated in microwave at 80^{o}C for 1 h (TLC), solvent was removed under reduced pressure and residue was diluted with DCM (20 ml) and washed with 1N HCl (1×15ml). This aqueous layer was basified with saturated solution of NaOH up to pH 12 and extracted with DCM (3×15ml). Organic phase was dried over $MgSO_4$ and solvent was removed under reduced pressure to obtain compound **9a-b**.

N-(4-((diethylamino)methyl)-3-hydroxyphenyl)acetamide **9a:** Yellow oil (77%); R_f 0.2 (5% MeOH-DCM); ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.72 (br s, 1H), 7.04 (d, J = 1.7 Hz, 1H), 6.91 (m, 2H), 3.65 (s, 2H), 2.54 (q, J = 8.0 Hz, 4H), 2.01 (s, 3H), 1.02 (t, J = 7.1 Hz, 6H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 168.4, 158.1, 139.7, 128.9, 117.9, 109.9, 106.8, 55.3 (2C), 46.4, 46.1, 24.4 and 11.5; MS: *m/z* 237 $[M+H]^+$.

N-(3-((Diethylamino)methyl)-4-hydroxyphenyl)acetamide **9b:** Yellow oil (77%); R_f 0.2 (5% MeOH-DCM); ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.61 (br s, 1H), 7.29 (d, J = 2.4 Hz, 1H), 7.26 (dd, J = 8.6, 2.5 Hz, 1H), 6.62 (d, J = 8.5 Hz, 1H), 3.67 (s, 2H), 2.55 (q, J = 7.2 Hz, 4H), 1.98 (s, 3H), 1.03 (t, J = 7.1 Hz, 6H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 167.9, 153.7, 131.3, 123.0, 120.5, 119.9, 115.5, 55.7 (2C), 46.5, 46.3, 24.1 and 11.5; MS: *m/z* 237 $[M+H]^+$.

General procedure for the synthesis of compound **10**

Compound **9a-b** (3 mmol) was refluxed in 6N HCl (3 ml) at 100°C for 2h (TLC). Solvent was removed under reduced pressure and residue was dissolved in EtOH (2×15 ml) and solvent was removed under reduced pressure to obtain product **10a-b** as a semisolid.

5-Amino-2-((diethylamino)methyl)phenol **10a:** Viscous solid (93%); R_f 0.2 (20% MeOH-DCM); ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.40 (br s, 2H), 10.11 (br s, 1H), 7.56 (d, J = 2.6 Hz, 1H), 7.34 (dd, J = 8.7, 2.7 Hz, 1H), 7.17 (d, J = 8.7 Hz, 1H), 4.23 (s, 2H), 3.08 (q, J = 8.0 Hz, 4H), 1.28 (t, J = 7.1 Hz, 6H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 156.9, 128.0, 126.4, 123.1, 117.8, 117.0, 49.6, 46.8 (2C) and 9.0 (2C); MS: *m/z* 195 $[M+H]^+$.

4-Amino-2-((diethylamino)methyl)phenol **10b:** Viscous solid (88%); R_f 0.2 (20% MeOH-DCM); ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.40 (s, 1H), 7.71 (s, 2H), 7.56 (d, J = 2.6 Hz, 1H), 7.34 (dd, J = 8.7, 2.7 Hz, 1H), 7.16 (d, J = 8.7 Hz, 1H), 4.23 (s, 2H), 3.06 (q, J = 8.0 Hz, 4H), 1.27 (t, J = 7.1 Hz, 6H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 156.9, 128.0, 126.4, 123.1, 117.8, 117.0, 56.4 (2C), 46.8, 18.9 and 9.0; MS: *m/z* 195 $[M+H]^+$.

2-Fluoro-5-nitrobenzyl 4-methylbenzenesulfonate **13:** To a solution of NaOH (0.27g, 6.72 mmoles) in H_2O (2 ml) was added a solution of 2-fluoro-5-nitro-phenyl)-methanol **11** (0.5g, 2.92 mmoles) in THF (10 ml) at 0°C. A solution of TsCl (0.95g, 4.97 mmoles) in THF (5 ml) is then added drop wise. The reaction mixture was stirred for 2h at 5-10 °C. 25 ml of water was added and the compound was extracted with DCM (3×20 mL). The organic layer was dried over MgSO4, filtered, evaporated. Expected compound was purified by column chromatography using hexane:ethylacetate as a eluent and title product was obtained as a white solid (0.9g, 95%); m. p. 89-91 °C; R_f 0.5 (30% EtOAc-Hexane); 1H NMR (300 MHz, $CDCl_3$) δ 8.22 (m, 2H), 7.82 (d, J = 8.5 Hz, 2H), 7.37 (d, J = 8.6 Hz, 2H), 7.21(d, J = 8.7 Hz, 1H), 5.19 (s, 2H), 2.46 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 165.1, 145.5 (2C), 144.2, 132.6, 130.0 (2C), 128.0 (2C), 126.6, 126.2, 116.8, 64.0 and 21.6; MS: *m/z* 326 $[M+1]^+$.

N-ethyl-N-(2-fluoro-5-nitrobenzyl)ethanamine **14:** To a solution of compound **13** (0.5g, 1.54 mmoles) in 1 ,4-dioxane (5 ml) was added TEA (0.32 ml, 2.31 mmoles) and diethylamine (0.24 ml, 2.31 mmoles). The reaction mixture was stirred for 4h at 60 °C, concentrated and purified by column chromatography using hexane:ethylacetate as a eluent. The expected compound was obtained as yellow oil (0.28g, 82% yield); R_f 0.7 (50% EtOAc-Hexane); 1H NMR (400 MHz, $CDCl_3$) δ 8.44 (dd, *J* = 6.3, 2.9 Hz, 1H), 8.12 (d, *J* = 2.9 Hz, 1H), 7.15 (d, *J* = 8.9 Hz, 1H), 3.67 (s, 2H), 2.58 (q, *J* = 7.1 Hz, 4H), 1.08 (t, *J* = 7.1 Hz, 6H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 167.4, 143.2, 126.8, 126.3, 124.0, 116.1, 54.6, 49.0 (2C) and 13.3 (2C); MS: *m/z* 227 $[M+1]^+$.

3-((Diethylamino)methyl)-4-fluoroaniline **15:** To a solution of compound **14** (0.25 mg, 1.10 mmoles) in THF (20 ml) was added a solution of $SnCl_2$ (0.84 mg, 4.42 mmoles) in THF (10 ml) and 1N HCl (3.4 mL). The reaction mixture was heated at 60 °C for 8h and concentrated. Saturated solution of $NaHCO_3$ was added until pH8. Aqueous layer was extracted with DCM (3×15 mL). The organic layer was dried over MgSO4, filtered, evaporated and obtained as yellow oil (0.2 mg, 92% yield); R_f 0.4 (20% MeOH-DCM); 1H NMR (400 MHz, $CDCl_3$) δ 6.80 (dd, *J* = 2.7, 8.7 Hz, 1H), 6.75 (d, *J* = 3.0 Hz, 1H), 6.49 (d, *J* = 8.7 Hz, 1H), 3.57 (s, 2H), 2.57 (q, *J* = 7.2 Hz, 4H), 1 .07 (t, *J* = 7.2 Hz, 6H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 150.3, 142.6, 125.2, 116.4, 115.3, 115.0, 55.6, 48.6 (2C) and 13.6 (2C); MS: *m/z* 197 $[M+1]^+$.

2-((ethylamino)methyl)-4-nitrophenol **17:** To a solution of 2-hydroxy-5-nitrobenzaldehyde **16** (1.0g, 5.98 mmol) in dry MeOH (50 ml) at 25^{o}C was added ethylamine (0.67 ml, 11.96 mmoles) dropwise. The mixture was allowed to stir for 1 h after which the temperature was reduced to 0^{o}C in an ice bath and $NaBH_4$ was added in small portions over 45 min. The reaction mixture was allowed to stir for 16 h. Water (15 ml) was added and the solvent was removed under reduced pressure. The residue was dissolved in DCM and filtered through celite. The solvent was removed *in vacuo* to obtain orange solid (89%); m.p. 182-184 ^{o}C; R_f

0.4 (20% MeOH-DCM); ^{1}H NMR (400 MHz, DMSO) δ 8.40 (d, *J* = 2.9 Hz, 1H), 8.13 (dd, *J* = 2.9, 9.1 Hz, 1H), 7.18 (d, *J* = 9.1 Hz, 1H), 4.11 (s, 2H), 2.96 (q, *J* = 7.2 Hz, 2H), 1.24 (t, *J* = 7.2 Hz, 3H); ^{13}C NMR (101 MHz, DMSO) δ 163.6, 138.4, 127.7, 126.4, 119.4, 115.9, 43.9, 41.8 and 10.8; MS: *m/z* 197.1 $[M+H]^+$.

4-amino-2-((ethylamino)methyl)phenol **18:** To a solution of **17** (0.5g, 2.55 mmol) in dry MeOH (100 ml) at 25^{o}C was added palladium on carbon catalyst in small portions. The mixture was allowed to stir under hydrogen gas atmosphere for 16 h. The reaction mixture was filtered through celite and the solvent removed under reduced pressure to obtain brown viscous oil (85%); R_f 0.2 (20% MeOH-DCM); ^{1}H NMR (400 MHz, DMSO) δ 6.70 (d, *J* = 8.5 Hz, 1H), 6.60 (d, *J* = 2.7 Hz, 1H), 6.52 (dd, *J* = 2.7, 8.5 Hz, 1H), 3.90 (s, 2H), 2.90 (q, *J* = 7.2 Hz, 2H), 1.21 (t, *J* = 7.2 Hz, 3H); ^{13}C NMR (101 MHz, DMSO) δ 146.9, 140.4, 118.4, 117.0, 116.5, 116.4, 44.8, 41.4 and 10.7; MS: *m/z* 167 $[M+H]^+$.

General procedure for the synthesis of compound **19a-f and 19i**

Compound **10a-b/15/18** (1.0 equiv.) was added to a stirred mixture of compound **6a-d** (1.1 equiv.) and triethylamine (4.0 equiv.) in THF:DMF (4.5:0.5) in microwave tube. The mixture was irradiated in microwave at 80^{o}C for 20 min. The solvent was removed under reduced pressure and the residue was purified through column chromatography using MeOH:DCM as a eluent. The compound was further recrystallized from ethanol.

General procedure for the synthesis of compound **19g-h**

Compounds **6b-c** (1.0 equiv.) was added to a stirred mixture of **18** (1.5 equiv.) in EtOH in a microwave tube followed by HCl (50 µL, 4M HCl in dioxane). The reaction mixture was irradiated in microwave at 80^{o}C for 1.3 h. The solvent was evaporated *in vacuo* and the residue was purified by flash chromatography using 5 – 20% MeOH/DCM as eluent to obtain the desired compounds.

1-((3-((Diethylamino)methyl)-4-hydroxyphenyl)amino)-3-(4-(trifluoromethyl)phenyl)benzo[4,5]imidazo[1,2-*a*]pyridine-4-carbonitrile **19a:**

Yellow solid (10%); m. p. 151-153 ^{o}C; R_f 0.3 (6% MeOH-DCM); ^{1}H NMR (600 MHz, DMSO) δ 8.71 (d, *J* = 8.2 Hz, 1H), 7.85 (d, *J* = 8.2 Hz, 2H), 7.76 (d, *J* = 8.2 Hz, 2H), 7.71 (d, *J* = 7.8 Hz, 1H), 7.46 (t, *J* = 7.5 Hz, 1H), 7.25 (t, *J* = 7.6 Hz, 1H), 7.12 (s, 1H), 7.08 (d, *J* = 8.4 Hz, 1H), 6.84 (d, *J* = 8.4 Hz, 1H), 5.79 (s, 1H), 3.92 (s, 2H), 2.77 (q, *J* = 7.1 Hz, 4H), 1.09 (t, *J* = 7.1 Hz, 6H); ^{13}C NMR (150 MHz, DMSO) δ 150.9, 150.8, 150.5 (2C), 148.4 (2C), 144.8, 144.7, 142.9 (2C), 129.8, 129.3 (2C), 125.9 (2C), 125.8, 125.4, 124.9, 123.6, 121.8, 119.9, 117.2, 116.7 (2C), 93.5, 53.3, 46.7 (2C) and 10.5 (2C); MS: *m/z* 530 $[M+H]^+$; HPLC purity 99% (t_r = 3.44).

1-((4-((Diethylamino)methyl)-3-hydroxyphenyl)amino)-3-(trifluoromethyl)benzo[4,5]imidazo[1,2-*a*]pyridine-4-carbonitrile **19b:**

Yellow solid (0.2g, 53%); m. p. 314-316 ^{o}C; R_f 0.3 (8% MeOH-DCM); ^{1}H NMR (400 MHz, CD_3OD) δ 8.78 (d, *J* = 8.3 Hz, 1H), 7.61 (d, *J* = 7.9 Hz, 1H), 7.40 (t, *J* = 7.6 Hz, 1H), 7.26 (d, *J* = 7.9 Hz, 1H), 7.19 (t, *J* = 7.3 Hz, 1H), 6.64 (d, *J* = 1.9 Hz, 1H), 6.61 (dd, *J* = 7.9, 2.0 Hz, 1H), 6.03 (s, 1H), 4.25 (s, 2H), 3.20 (q, *J* = 7.3 Hz, 4H), 1.37 (t, *J* = 7.3 Hz, 6H); ^{13}C NMR (100 MHz, CD_3OD) δ 157.7, 153.0, 151.5, 150.2, 135.9, 132.3, 130.9, 130.2, 128.4, 124.6, 121.8, 119.6, 117.4, 116.6, 114.6, 113.8, 110.8, 108.7, 90.8, 67.7, 52.2 (2C) and 12.9 (2C); MS: *m/z* 454 $[M+H]^+$; HPLC purity 99% (t_r = 3.50).

1-((3-((Diethylamino)methyl)-4-hydroxyphenyl)amino)-3-(trifluoromethyl)benzo[4,5]imidazo[1,2-*a*]pyridine-4-carbonitrile **19c:**

Yellow solid (0.12g, 26%); m. p. 172-174 ^{o}C; R_f 0.3 (6% MeOH-DCM); ^{1}H NMR (400 MHz, DMSO) δ 8.81 (d, *J* = 7.6 Hz, 1H), 7.56 (d, *J* = 7.9 Hz, 1H), 7.34 (t, *J* = 8.0 Hz, 1H), 7.11 (t, *J* = 8.0 Hz, 1H), 7.01 (d, *J* = 2.0 Hz, 1H), 6.92 (m, 2H), 5.78 (s, 1H), 4.19 (s, 2H), 3.09 (q, *J* = 7.1 Hz, 4H), 1.24 (t, *J* = 7.2 Hz, 6H); ^{13}C NMR (100 MHz, DMSO) δ 152.0, 151.8, 150.9,

145.0, 142.8, 134.7, 134.4, 130.9, 125.7, 125.3, 124.8, 124.5, 122.6, 119.1, 118.6, 118.2, 117.0, 116.9, 116.1, 88.7, 51.4, 47.1 and 9.2 (2C); MS: *m/z* 454 $[M+H]^+$; HPLC purity 99% (t_r = 3.29).

1-((3-((diethylamino)methyl)-4-hydroxyphenyl)amino)-7,8-difluoro-3-(4-(trifluoromethyl)phenyl)benzo[4,5]imidazo[1,2-*a*]pyridine-4-carbonitrile **19d:**

Yellow solid (26 %); m.p 284-288 °C; R_f 0.65 (20 % MeOH-DCM); ^{1}H NMR (400 MHz, DMSO) δ 8.76 (dd, *J* = 11.5, 8.7 Hz, 1H), 7.80 (d, *J* = 8.2 Hz, 2H), 7.69 (d, *J* = 8.2 Hz, 2H), 7.55 (dd, *J* = 11.5, 8.7 Hz, 1H), 7.06 (d, *J* = 2.6 Hz, 1H), 6.99 (dd, *J* = 8.5, 2.6 Hz, 1H), 6.86 (d, *J* = 8.5 Hz, 1H), 5.59 (s, 1H), 4.08 (s, 2H), 2.97 (q, *J* = 7.2 Hz, 4H), 1.16 (t, *J* = 7.2 Hz, 6H); ^{13}C NMR (101 MHz, DMSO) δ 153.8, 151.0, 149.3, 149.2, 147.8, 146.8, 144.9, 143.8, 141.8, 141.6, 129.4, 129.2, 126.2, 126.0, 125.8, 125.8, 125.1, 123.3, 120.3, 116.7, 105.0, 104.8, 103.5, 103.3, 92.6, 55.4, 51.9, 47.0, 25.6 and 9.6; HPLC-MS (APCI/ESI): Purity= 97%, t_R = 4.15 min, MS: *m/z* 566.2 $[M+H]^+$.

1-((3-((diethylamino)methyl)-4-hydroxyphenyl)amino)-2-fluoro-3-(4-(trifluoromethyl)phenyl)benzo[4,5]imidazo[1,2-*a*]pyridine-4-carbonitrile **19e**:

Yellow solid (11 %); m.p 275-280 °C; R_f 0.49 (20 % MeOH-DCM); ^{1}H NMR (400 MHz, DMSO) δ 8.66 (d, 1H), 7.88 (d, *J* = 8.1 Hz, 2H), 7.74 (d, *J* = 8.1 Hz, 2H), 7.59 (d, *J* = 7.8 Hz, 1H), 7.37 (t, *J* = 7.8 Hz, 1H), 7.10 (t, *J* = 7.8 Hz, 1H), 6.92 – 6.81 (m, 2H), 6.75 (d, *J* = 8.5 Hz, 1H), 4.02 (s, 2H), 2.92 (q, *J* = 8.0 Hz, 4H), 1.15 (t, *J* = 8.0 Hz, 3H); ^{13}C NMR (101 MHz, DMSO δ 152.4, 151.4, 149.3, 144.8, 144.7, 142.8, 137.8, 131.5, 130.9, 129.7, 129.4, 126.0, 125.6 (2C), 124.9, 123.5, 123.3, 122.5, 119.8, 118.9, 117.1, 116.2, 115.7, 106.5, 98.3, 52.1, 46.7, 31.1, 29.8 and 9.6; HPLC-MS (APCI/ESI): Purity= 99%, t_R = 3.98 min, MS: *m/z* 548.1 $[M+H]^+$.

1-((3-((Diethylamino)methyl)-4-fluorophenyl)amino)-3-(trifluoromethyl)benzo[4,5]imidazo[1,2-*a*]pyridine-4-carbonitrile **19f:**

Yellow solid (33%); m. p. 209-211 ^{o}C; R_f 0.2 (5% MeOH-DCM); ^{1}H NMR (400 MHz, DMSO) δ 8.81 (d, *J* = 7.6 Hz, 1H), 7.58 (d, *J* = 7.8 Hz, 1H), 7.37 (t, *J* = 8.0 Hz, 1H), 7.31 (t, *J* = 8.0 Hz, 1H), 7.21 (dd, *J* = 6.7, 2.5 Hz, 1H), 7.12 (m, 2H), 5.76 (s, 1H), 4.29 (s, 2H), 3.11 (q, *J* = 7.1 Hz, 4H), 1.24 (t, *J* = 7.2 Hz, 6H); ^{13}C NMR (100 MHz, DMSO) δ 155.7, 151.4, 150.7, 135.0, 134.9, 130.6, 126.2, 125.4, 125.2, 124.7 (2C), 119.3 (2C), 118.4, 117.1 (2C), 116.9, 116.1, 88.3, 49.3, 47.3 (2C) and 8.9 (2C); MS: *m/z* 456 $[M+H]^+$; HPLC purity 99% (t_r = 4.23).

1-((3-((ethylamino)methyl)-4-hydroxyphenyl)amino)-7,8-difluoro-3-(4-(trifluoromethyl)phenyl)benzo[4,5]imidazo[1,2-a]pyridine-4-carbonitrile **19g**: Yellow solid (11%); m. p. 232-234 ^{o}C; R_f 0.2 (15% MeOH-DCM); ^{1}H NMR (400 MHz, DMSO) δ 8.76 (dd, *J* = 11.5, 8.7 Hz, 1H), 7.80 (d, *J* = 8.2 Hz, 2H), 7.69 (d, *J* = 8.2 Hz, 2H), 7.55 (dd, *J* = 11.5, 8.7 Hz, 1H), 7.06 (d, *J* = 2.6 Hz, 1H), 6.99 (dd, *J* = 8.5, 2.6 Hz, 1H), 6.86 (d, *J* = 8.5 Hz, 1H), 5.59 (s, 1H), 4.08 (s, 2H), 2.97 (q, *J* = 7.2 Hz, 2H), 1.16 (t, *J* = 7.2 Hz, 3H); ^{13}C NMR (101 MHz, DMSO) δ 153.8, 151.0, 149.3 (2C), 147.8, 146.8, 144.9, 143.8, 141.8 (2C), 141.6, 129.4, 129.2 (2C), 126.0, 125.8 (2C), 125.1, 123.3, 120.3, 116.7, 105.0, 104.8, 103.5, 103.3, 92.6, 55.4, 47.0 and 9.6; MS: *m/z* 538 $[M+H]^+$; HPLC purity 99% (t_r = 2.70).

1-((3-((ethylamino)methyl)-4-hydroxyphenyl)amino)-3-(4-(trifluoromethyl)phenyl)benzo[4,5]imidazo[1,2-a]pyridine-4-carbonitrile **19h**: Yellow solid (14%); m. p. 230-232 ^{o}C; R_f 0.2 (15% MeOH-DCM); ^{1}H NMR (600 MHz, DMSO) δ 8.67 (d, *J* = 8.3 Hz, 1H), 7.85 (m, 4H), 7.78 (d, *J* = 8.1 Hz, 1H), 7.53 (t, *J* = 7.8 Hz, 1H), 7.43 (m, 1H), 7.33 (t, *J* = 7.8 Hz, 1H), 7.22 (m, 1H), 7.07 (d, *J* = 8.5 Hz, 1H), 5.94 (s, 1H), 4.03 (s, 2H), 2.90 (q, *J* = 7.3 Hz, 2H), 1.21 (t, *J* = 7.3 Hz, 3H); ^{13}C NMR (151 MHz, DMSO) δ 150.9,

150.8, 150.5 (2C), 148.4 (2C), 144.8 (2C), 142.9 (2C), 129.8, 129.3 (2C), 125.9 (2C), 125.8, 125.4, 124.9, 123.6, 121.8, 119.9, 117.2, 116.7 (2C), 93.5, 53.3, 46.7 and 10.5; MS: *m/z* 502 $[M+H]^+$; HPLC purity 99% (t_r = 2.60).

1-((4-((diethylamino)methyl)-3-hydroxyphenyl)amino)-3--(4-(trifluoromethyl)phenyl)benzo[4,5]imidazo[1,2-*a*]pyridine-4-carbonitrile **19i**: Yellow solid (20%); m. p. 232-234 ^oC; R_f 0.2 (10% MeOH-DCM); ^1H NMR (600 MHz, CD_3OD) δ 8.57 (d, *J* = 8.3 Hz, 1H), 7.75 (m, 4H), 7.72 (d, *J* = 7.9 Hz, 1H), 7.46 (t, *J* = 7.6 Hz, 1H), 7.26 (d, *J* = 7.9 Hz, 1H), 7.25 (t, *J* = 7.3 Hz, 1H), 7.04 (d, *J* = 7.8 Hz, 1H), 6.65 (m, 2H), 6.11 (s, 1H), 3.86 (s, 2H), 2.75 (q, *J* = 7.3 Hz, 4H), 1.15 (t, *J* = 7.3 Hz, 6H); ^{13}C NMR (151 MHz, CD_3OD) δ 159.8, 149.7, 149.5, 145.5 (2C), 144.2, 131.8, 131.3, 131.2, 130.5, 129.8 (2C), 126.1 (2C), 124.6, 123.2, 121.8, 120.1, 117.6, 117.1, 116.7, 114.5, 110.8, 108.7, 90.8, 56.6, 47.2 (2C), and 11.1 (2C); MS: *m/z* 530 $[M+H]^+$; HPLC purity 98% (t_r = 2.62).

MATERIALS AND METHODS:

In Vitro *P. falciparum* Assay and In Vivo Antimalarial Efficacy Studies*:* Compounds were screened against multidrug resistant (*Pf*K1) and sensitive (*Pf*NF54) strains of *P. falciparum* in vitro using the modified [^3H]-hypoxanthine incorporation assay.[26] In vivo efficacy against *P. berghei* was performed using mice (n = 3) which were infected with a GFP-transfected *P. berghei* ANKA strain (donated by A. P. Waters and C. J. Janse, Glasgow and Leiden Universities respectively).[27] Parasitemia was determined using standard flow cytometry techniques. Briefly, 3 μL of tail blood from mice in the treated and control groups was pipetted into staining solution of 100 nM MitoTracker Deep Red and 1× SYBR green in 1× phosphate-buffered saline (pH 7.4) and incubated at 37 °C for at least 20 min in the dark. The cells were then quantified on an Accuri C6 flow cytometer (Becton Dickinson) with

approximately 10, 000 events read per sample. The detection limit was 1 parasite per 1,000 erythrocytes (that is, 0.1%). Activity was calculated as the difference between the mean % parasitemia for the control and treated groups expressed as a % relative to the control group. Compounds were dissolved or suspended in 90/10 Tween80/ethanol (v/v), diluted 10 times with water and administered orally as four consecutive daily doses (4, 24, 48 and 72 h after infection). Animals were considered cured if there were no detectable parasites on day 30 post-infection as determined by light microscopy.

Cytotoxicity Testing: Compounds were screened for *in vitro* cytotoxicity against Chinese Hamster Ovarian (CHO) mammalian cell-lines, using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT)-assay. The reference standard, emetine, was prepared to 2 mg/ml in distilled water while stock solutions of test compounds were prepared to 20 mg/ml in 100% DMSO with the highest concentration of solvent to which the cells were exposed having no measurable effect on the cell viability. The initial concentration of the drugs and control 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. Plates were incubated for 48 h with 100 μL of drug and 100 μL of cell suspension in each well and developed afterwards by adding 25 μL of sterile MTT (Thermo Fisher Scientific) to each well and followed by 4 h incubation in the dark. The plates were then centrifuged, medium aspirated off and 100 μL of DMSO added to dissolve crystals before reading absorbance at 540 nm. IC_{50} values were then obtained from dose-response curves, using a nonlinear dose-response curve fitting analysis via GraphPad Prism v.4.0 software (La Jolla, USA). The assay was conducted in triplicate and conducted on two separate occasions.

Single Point In Vitro Metabolic Stability Assay: The test compound (1 μM) was incubated at 37 °C in a solution containing 0.35 mg/mL microsomes (MLM; male Mouse BALB/c,

Xenotech, or HLM, mixed gender, Xenotech) and metabolic reactions were initiated by the addition of NADPH (1 mM) in phosphate buffer (100 mM, pH 7.4), and incubated for 30 min. The samples were then prepared by cold-ice acetonitrile precipitation containing 0.1 μM carbamazepine (internal standard), centrifuged and filtered for LC-MS analysis. The incubation of compounds and controls (midazolam and MMV390048) were performed in triplicate. The relative loss of parent compound over the course of the incubation was monitored by LC-MS/MS and results reported as % remaining after 30 min incubation.

LC-MS/MS analyses were performed on a 4000 QTRAP® mass spectrometer (AB Sciex) equipped with a Turbo V™ ion source and coupled to an Agilent 1200 Rapid Resolution HPLC system (600 bar, Agilent technologies, USA). 2 μL of samples stored in a sample tray set at 8°C were injected onto a Kinetex C18 column, 2.1 mm x 50 mm, 2.6 μm particles (Phenomenex) or Kinetex PFP column, 2.1 mm x 50 mm, 2.6 μm particles (Phenomenex) at 40 °C. Compounds were separated using a gradient solvent system consisting of 0.1% formic acid (A) and 0.1% formic acid in acetonitrile (B). MRM scans were operated under electrospray positive ionization mode and the operation parameters were as follows: curtain gas, 30 psi; nebulizer gas (GS1), 50 psi; turbo gas (GS2), 60 psi; source temperature, 500 °C; ion spray voltage, 5000 V; declustering potential and collision energy optimized from infusion of the compounds; collisional activated dissociation (CAD) gas setting: medium.

Metabolite Identification and Glutathione Trapping Experiments: Metabolites formed in the incubations to determine single point microsomal stability were identified by comparison of their respective chromatograms with chromatograms at T=0 and in the buffer only control using Lightsight v2.3. The tentative identity of the metabolites was deduced by comparison of the product ion spectra of the $[M+H]^+$ ions of the metabolites with that of parent

compound. A confirmatory identification was done by comparing the retention and fragmentation of the detected metabolite to that of a synthetic metabolite.

For the glutathione (GSH) trapping experiments, the test compounds (10 μM) were incubated at 37 °C in solutions containing HLM (1 mg/mL; mixed gender, pool of 50 individuals, Xenotech, Lot. 1210153), reduced glutathione (5 mM, Sigma Aldrich) and NADPH (1 mM, Calbiochem) in sodium phosphate buffer (50 mM, pH 7.4). Control incubations in which either GSH, NADPH, test compound or HLM were individually excluded were also prepared. For each test and control preparation, T0 incubations were terminated by addition of acetonitrile at the start of the incubation. T90 incubations were terminated after 90 minutes by addition of ice-cold acetonitrile. All samples were subsequently centrifuged at 10,000 rpm for 10 min and the resultant supernatant solutions filtered for LC-MS analysis. Diclofenac (5 μM) was incubated concomitantly as a positive control. The supernatant was analyzed by LC-MS/MS (5500 Q-TRAP AB Sciex) with a Supelco Ascentis C18, 4.6x150 mm, 2.6 μm particles column, using EMS-IDA-EPI methods.

ASSOCIATED CONTENT

Supporting Information

Additional details of the structures of all derivatives assessed are provided as Supplementary material. Excel file with the compounds SMILES format is also provided

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FIGURES AND TABLES

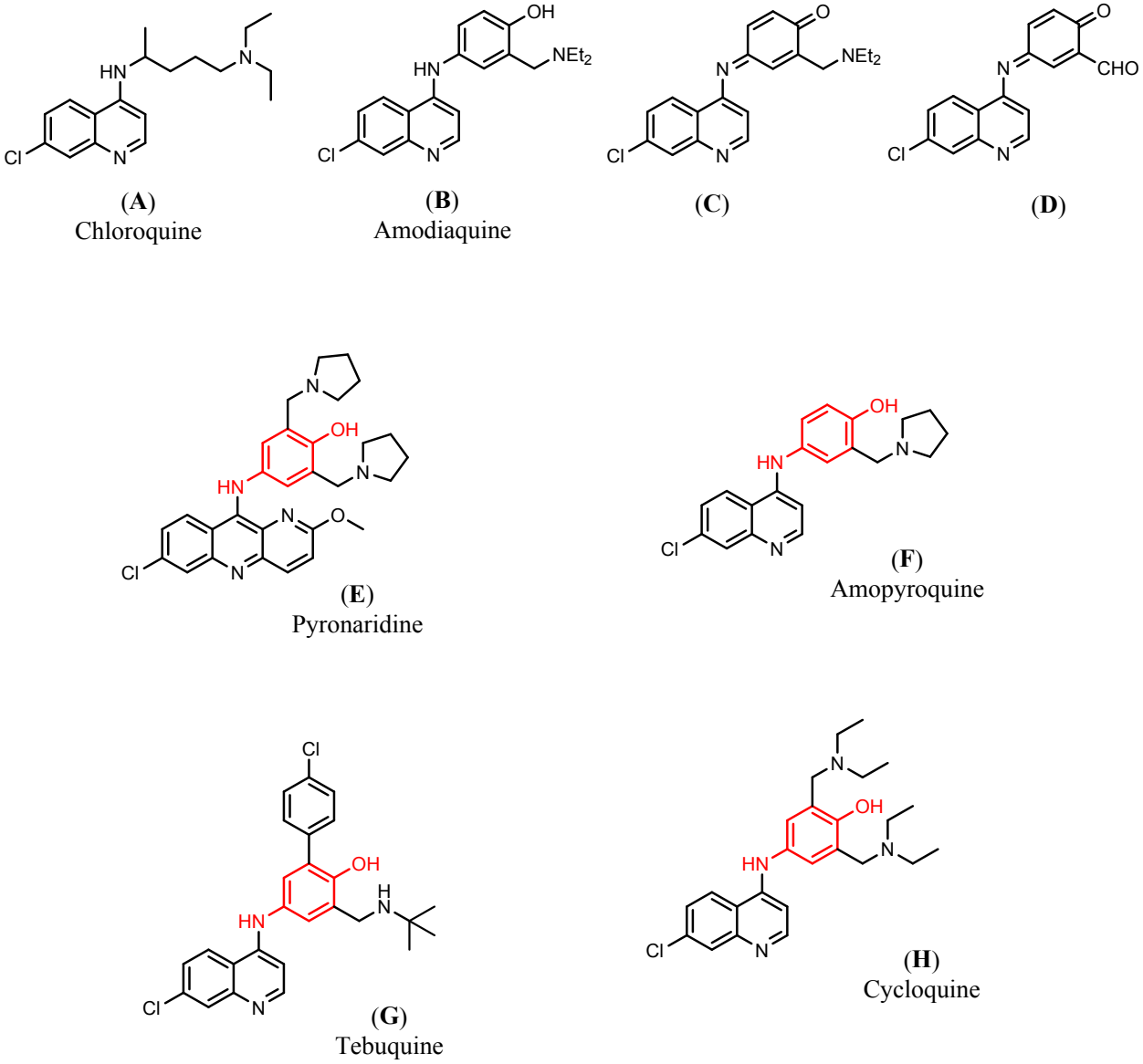
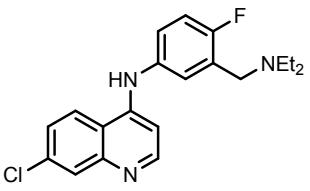
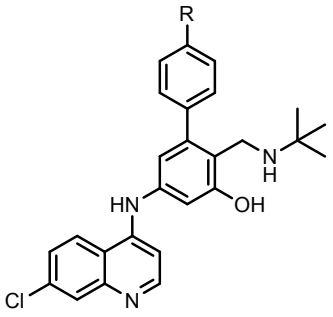


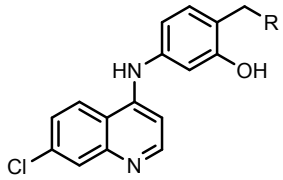
Figure 1: Chemical structures of chloroquine (**A**), amodiaquine (**B**), reactive amodiaquine metabolites (**C** and **D**) and examples of antimalarials in clinical use or under development (**E** – **H**) that bear the 4-aminophenol motif (highlighted in red).



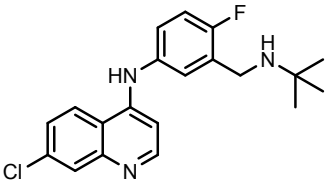
(**I**)
4'-dehydroxy-4'-fluoroamodiaquine



(**J**)
Isotebuquine derivatives

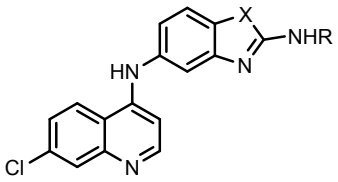


(**K**) Isoquine R = NEt_2



(**M**)
4'-fluoro-*N*-*tert*-butyl amodiaquine

(**L**) N-*tert*-butyl isoquine R = NHtBu



(**N**) Benzothiazole X = S; R = $-C(CH_3)_3$ or $-CH_2CH_3$

(**O**) Benzoxazole X = O; R = $-C(CH_3)_3$ or $-CH_2CH_3$

(**P**) Benzimidazole X = NH; R = $-C(CH_3)_3$ or $-CH_2CH_3$

Figure 2: Chemical structures of representative examples of compounds (**I** – **P**) from different studies designed to bypass AQ-like bioactivation.

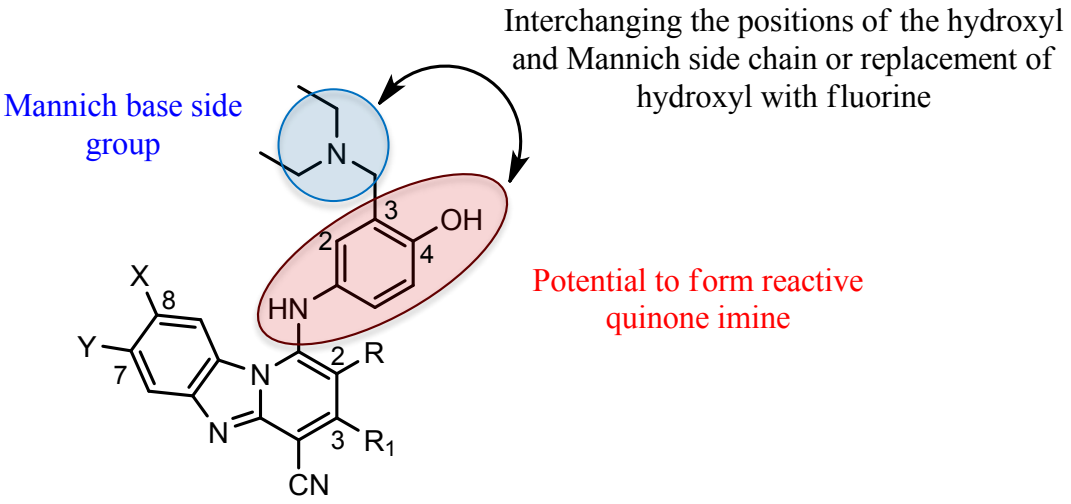


Figure 3: Chemical modifications around PBI scaffold to afford different Mannich base derivatives.

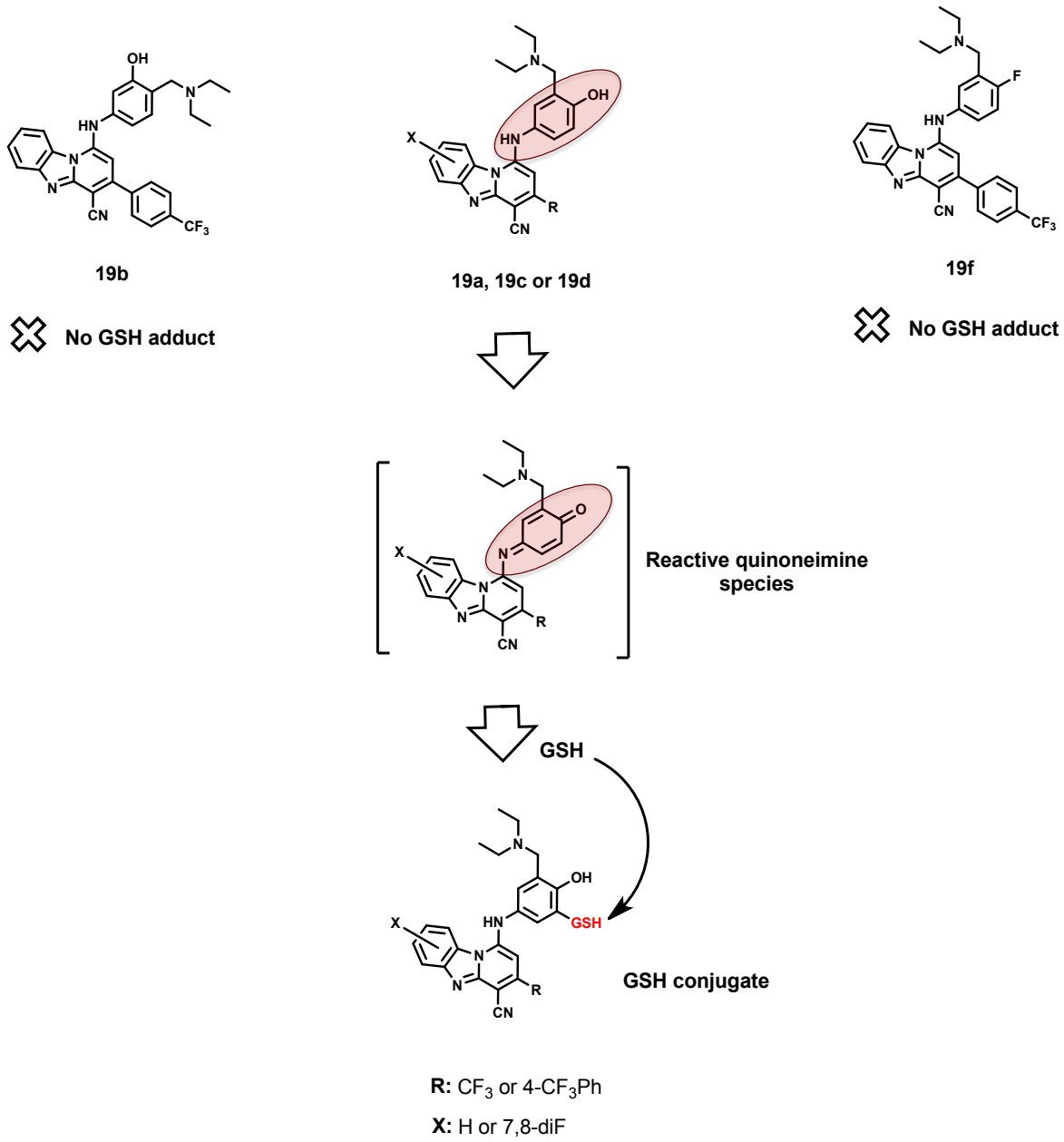
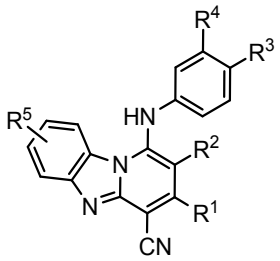


Figure 4: Proposed bioactivation of **19a**, **19c** and **19d**, and their putative conjugation with reduced GSH compared with the non-adduct forming **19b** and **19f**

TABLE 1: In vitro antiplasmodial activity and cytotoxicity of compounds **19a – 19i**



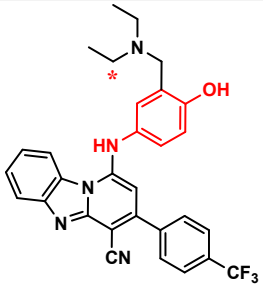
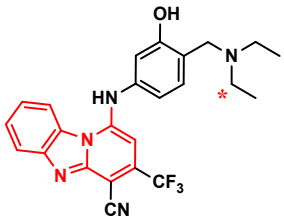
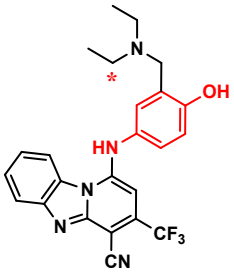
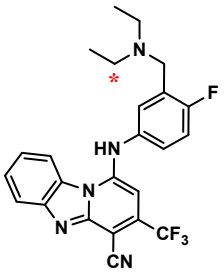
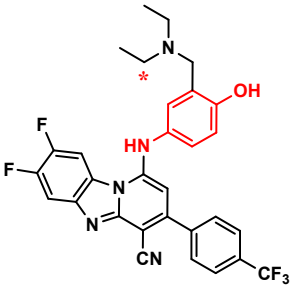
Code	R^1	R^2	R^3	R^4	R^5	[a]Antiplasmodium Activity (μM)			Cytotoxicity	
						*Pf*NF54 IC_{50}	*Pf*K1 IC_{50}	R.I	CHO IC_{50}	S.I
19a	4-CF_3Ph	H	OH	$CH_2N(CH_2CH_3)_2$	H	0.11	0.18	1.6	189	1718
19b	CF_3	H	$CH_2N(CH_2CH_3)_2$	OH	H	4.00	3.81	1.0	173	43
19c	CF_3	H	OH	$CH_2N(CH_2CH_3)_2$	H	0.89	1.00	1.1	24	27
19d	4-CF_3Ph	H	OH	$CH_2N(CH_2CH_3)_2$	7,8-diF	0.07	0.07	1.0	>100	1429
19e	4-CF_3Ph	F	OH	$CH_2N(CH_2CH_3)_2$	H	0.35			>100	286
19f	CF_3	H	F	$CH_2N(CH_2CH_3)_2$	H	5.14	4.66	0.9	142	28
19g	4-CF_3Ph	H	OH	$CH_2NH(CH_2CH_3)$	7,8-diF	0.08	0.17	2.1	>50	>625
19h	4-CF_3Ph	H	OH	$CH_2NH(CH_2CH_3)$	H	0.09	0.19	2.1	>50	>556
19i	4-CF_3Ph	H	$CH_2N(CH_2CH_3)_2$	OH	H	3.19	6.62	2.1	>50	>16
Chloroquine						0.02	0.27	16.8		
Emetine									0.095	

R. I = Resistance Index (*Pf*K1 IC_{50}/*Pf*NF54 IC_{50}); **S. I** = Selectivity Index (CHO IC_{50}/*Pf*NF54 IC_{50}).

[a]Mean of n = ≥2 independent [^{3}H]-hypoxanthine incorporation experiments.

CHO IC_{50}s denoting cytotoxicity are presented in micromolar (μM) units

TABLE 2: Metabolic stability and main biotransformation(s) of **19a – 19f**

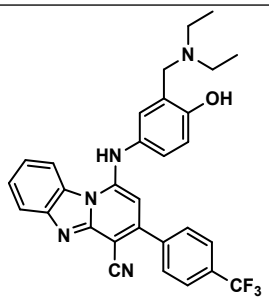
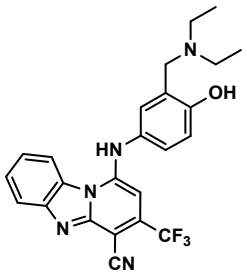
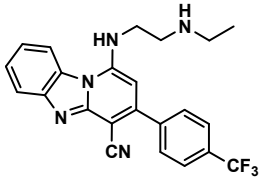
Compound	Structure and metabolic soft spots	% remaining ± SD after 30 min in MLM	Main biotransformation(s)*
19a		35 ± 3	*N*-deethylation, GSH adduct
19b		<10	*N*-deethylation, core oxidation
19c		15 ± 3	*N*-deethylation, GSH adduct
19f		<10	*N*-deethylation
19d		30.0 ± 9.4	*N*-deethylation, GSH adduct
Midazolam		<10	

Compound	Structure and metabolic soft spots	% remaining ± SD after 30 min in MLM	Main biotransformation(s)*
MMV390048		93.6	

SD: standard deviation

*Relative amounts calculated by LightSight as a percentage of total peak area (parent and metabolites), assuming similar MS response

TABLE 3: In vivo oral efficacy of **19a** and **19c** in *P. berghei*-infected mice

Compound	Structure	Antiplasmodial Activity		Oral Dose [a] (mg/kg)	% Reduction in Parasitemia	Mean Survival Days
		*Pf*NF54	*Pf*K1			
19a (KP84)		0.11	0.18	4 x 50 mg/kg	95	16
19c (KP94)		0.89	1.0	4 x 50 mg/kg	<40	4
KP4 [b]		0.12	0.11	4 x 50 mg/kg	96	14
Amodiaquine				4 x 50 mg/kg	99.9	30

Control	-	-	4 [c]

[a]Test compounds were formulated in 90/10 Tween80/ethanol (v/v), diluted 10 times with water and administered orally once per day on 4 consecutive days (4, 24, 48, and 72 h after infection).

[b]Used the HCl salt form.

[c]Mice with < 40 % parasitemia reduction were euthanized on day 4 in order to prevent death otherwise occurring at day 6.

FOR TABLE OF CONTENT USE ONLY

Block formation of reactive metabolite by interchanging the positions of the -OH and Mannich side chain or replacement of -OH with fluorine