Discovery and Preclinical Pharmacology of INE963, a Potent and Fast-Acting Blood-Stage Antimalarial with a High Barrier to Resistance and Potential for Single-Dose Cures in Uncomplicated Malaria

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ABSTRACT: A series of 5-aryl-2-amino-imidazothiadiazole (ITD) derivatives were identified by a phenotype-based high-throughput screening using a blood stage Plasmodium falciparum (Pf) growth inhibition assay. A lead optimization program focused on improving antiplasmodium potency, selectivity against human kinases, and absorption, distribution, metabolism, excretion, and toxicity properties and extended pharmacological profiles culminated in the identification of INE963 (1), which demonstrates potent cellular activity against Pf3D7 (EC50 = 0.006 μM) and achieves “artemisinin-like” kill kinetics in vitro with a parasite clearance time of <24 h. A single dose of 30 mg/kg is fully curative in the Pf-humanized severe combined immunodeficient mouse model. INE963 (1) also exhibits a high barrier to resistance in drug selection studies and a long half-life (T1/2) across species. These properties suggest the significant potential for INE963 (1) to provide a curative therapy for uncomplicated malaria with short dosing regimens. For these reasons, INE963 (1) was progressed through GLP toxicology studies and is now undergoing Ph1 clinical trials.

INTRODUCTION

Malaria is the world’s most prevalent infectious and life-threatening disease, caused by the protozoan parasite Plasmodium that is transmitted to human hosts by female Anopheles mosquitoes. In 2019, there were an estimated 229 million cases and 409,000 deaths, mostly affecting children aged under 5 years. Nearly half of the world’s population is at risk of malaria infection. Most malaria cases and deaths occur in sub-Saharan Africa, while South-East Asia, Eastern Mediterranean, Western Pacific, and the Americas are also at risk. Currently artemisinin-based combination therapy is recommended as a first-line treatment for uncomplicated malaria caused by Plasmodium falciparum (Pf) and has proved effective in reducing the disease burden.1

Artemisinin derivatives are highly potent and fast-acting antimalarials that produce rapid clinical responses to treatment. However, there has been an increasing number of reports of artemisinin-resistant malaria in the Greater Mekong subregion,2 and in addition, resistance against partner drugs such as mefloquine and piperaquine is already widespread.3,4 Furthermore, recent reports of dihydroartemisinin-piperaquine treatment failures have been documented.5 The emergence of
artemisinin resistance is a real threat to malaria treatment and elimination efforts worldwide. As a consequence, there is still an urgent need to identify new antimalarial compounds that can be added to or substitute the current artemisinin-based therapies. The artemisinin family has a short half-life and needs to be combined with a longer-acting partner drug for complete clearance of parasites. Therefore, our goal at the Novartis Institute for Tropical Diseases (NITD) is to identify a fast-acting antimalarial compound from a novel chemotype with activity against known resistance mutations and which will also have the potential for single-dose cures. Novel antimalarial chemotypes are expected to have a different mode of action to current drugs, and thus no cross resistance. In recent years, phenotype-based approaches have proven successful for the current drugs, and thus no cross resistance. In our continued research efforts at NITD to identify new antimalarial leads, our phenotypic screening campaigns and subsequent follow-up structure–activity relationship (SAR) studies around various hit series resulted in the identification of the 5-aryl-2-amino-imidazothiadiazole (ITD) lead compound, 2, which displayed modest potency (EC₅₀ of 0.27 μM) against the cultured parasite Pf 3D7 (Figure 1). During optimization of the scaffold, the late-lead compound 3 was rapidly identified with improved potency (EC₅₀ = 0.025 μM) and excellent in vivo pharmacokinetics. Compound 3 demonstrated a single dose ED₉₀ of 5.3 mg/kg against Pf infected mice and a similar speed-of-action to artemisinin. In addition, a single dose of 50 mg/kg was fully curative with no observed recrudescence after 60 days (Table 8). During our early studies of the 5-aryl-2-amino-imidazothiadiazole (ITD) chemical series, Medicines for Malaria Venture (MMV) independently conducted a high-throughput screen of a chemical library consisting of more than 250,000 compounds (MMV) and excellent distribution, metabolism, and excretion (ADME) properties suitable for a single-dose cure therapy in uncomplicated malaria.

**RESULTS AND DISCUSSION**

The proposed mechanism of tautomerization and chemical degradation suggested that removing the hydrogen of the 2-N(H)R linker would improve the chemical stability of the 5-aryl-2-amino-ITD scaffold. In addition, we had observed early on that related compounds which replaced the 2-N(H)R with a 2-N(Me)R linker were more chemically stable and maintained good potency. We were then pleased to find that the cyclic 4-methyl-4-amino-piperidine analog, 5, provided a margin to progress this compound further. Thus, to assess the broader human kinase inhibition profile of 3, the binding affinities to 468 human kinases were tested at a 10 μM concentration. Compound 3 inhibited 53 out of 468 human kinases at <10% of control (Figure 6), which we suspected was directly impacting the rat and dog toxicology findings and was likely a major factor resulting in the narrow safety margins observed.

Additionally, we observed that the in vitro potency of 3 decreased over time after multiple rounds of testing (EC₅₀ = 1.16 μM), indicating that 3 was likely degrading in DMSO stock solution. Chemical stability analysis revealed that the crystalline malate salt of 3 degraded over 24 h to 2.6% and 77.4% of the parent in pH 6.8 and fasted state simulated intestinal fluid (FaSSIF) solutions, respectively. Although 3 was chemically stable in the solid state, the solution instability was deemed a development risk. An additional analysis using 2D NMR elucidated the structure of the degraded product C/D, which suggested tautomerization of the 2-aminooITD moiety to the 2-imino-thiazolidine A. This tautomer A can be a trigger of nucleophilic cyclization by the terminal amine and result in spiro-cycle B, thereby leading to the degradation product C/D as illustrated in Figure 2.

![Figure 1. Early and late lead 5-aryl-2-amino-ITD antimalarial compounds discovered by Novartis and 5-aryl-2-amino-ITD hit disclosed by MMV.](image)

![Figure 2. Putative mechanism of tautomerization and degradation of 3.](image)
2.5-fold increase in antiplasmodium activity along with increased chemical stability. In addition, replacement of the 2-amino-linker with a 2-carbon-linker, 6 and 7, was also tolerated albeit with slightly lower potency. It is hypothesized that preventing tautomerization as well as the increasing rigidity of the side chain both improve chemical stability. Unexpectedly, replacement of the 2-amino-linker with an ether or thioether resulted in a dramatic loss in antiplasmodium activity. Considering the best potential to combine chemical stability, antiplasmodium potency, and synthetic tractability, 5 was selected as a starting point for further optimization (Table 1).

### Table 1. In Vitro Antiplasmodium Activity of 2-Amino and 2-Carbon Linked ITD Analogs

| cpd | R | $P_{EID7}$ EC$_{50}$ (μM)$^a$ | Chemical stability |
|-----|---|-------------------------------|--------------------|
| 3   | $^\cdot{-NH_2}$ | 0.025 | × |
| 5   | $^\cdot{-N}$ | 0.009 | √ |
| 6   | $^\cdot{-NH_2}$ | 0.033 (n=2) | √ |
| 7   | $^\cdot{-H}$ | 0.045 (n=1) | √ |

$^a$EC$_{50}$ values are given as average potency values in the Pf 3D7 assay ($n \geq 3$). Standard control melquine has an EC$_{50}$ value of 0.034 μM.

#### SARs for Pf 3D7 Activity and Selectivity against Human Haspin Kinase.

We first investigated the antiplasmodium SAR as well as the human kinase selectivity by systematic modifications of the 2,4-difluorobenzene moiety of 5 (Table 2). Haspin kinase proved to be the most sensitive and was used as the benchmark for selectivity comparisons. The IC$_{50}$ value of late lead compound 3 against Haspin was determined to be 0.011 μM. In addition, the screening hit 4 published by MMV, and our starting point 5, showed a similar Haspin inhibitory activity compared to 3. Replacement of the 2-F with 2-Me (8) and 2-OMe (9) reduced the Haspin inhibition to IC$_{50}$‘s of 1.28 and 1.74 μM, respectively, while maintaining antiplasmodium activity. Exchanging the 4-F to 4-OMe (10) decreased Haspin selectivity. Transposition of the 4-F to 6-F to give the 2,6-disubstituted analog (11) resulted in a 10-fold drop in antiplasmodium potency, presumably due to an increase in the biaryl dihedral angle (based on the observed orientation of compound 5 in the X-ray structure; Figure 3). The 3-OMe, 2-F analog (12) had a similar potency and selectivity as compared to 9, supporting the observation that the 2-OMe or 3-OMe substituent improves selectivity against Haspin. Replacement of the 2-OMe substituent with sterically larger groups such as OEt (13) and O-Pr (14) resulted in further reducing the Haspin inhibition while retaining potency. However, shifting the position of the ether oxygen of 13 to 2-CH$_2$OMe (15) decreased potency (EC$_{50}$ 0.11 μM). The 2-OCH$_2$CH$_2$OMe polar side chain (16) also led to weaker potency. The dihydrobenzofuran (17) had similar antiplasmodium potency and Haspin inhibitory activity to 9, while the indole (18) and N-methylindole analog (19) were less potent. Introduction of the pyridine nitrogen to 9 yielded the 2-fluoro-6-OMe pyridine (20), which had similar antiplasmodium activity but 2–3 fold lower selectivity against Haspin. The regioisomeric 2-OMe-pyridine analog (21) further decreased selectivity against Haspin. Heteroaromatics such as pyrimidine and thiadiazole were significantly less potent (22–26).

Several nonaromatic moieties were also examined. The tert-butylacetylene analog (27) had good antiplasmodium potency and maintained good selectivity against Haspin, however the cyclopropylacetylene (28) dropped the potency by 3-fold and the propargyl alcohol (29) was not tolerated. Cyclohexene (30) retained good antiplasmodium potency, but lost Haspin selectivity. Conversely, 4,4-difluorocyclohexane (31) and cyclopentane (32) moieties led to a significant loss of the antiplasmodium activity.

Compound 9 was then selected for in vitro profiling where it exhibited improved chemical stability in FaSSIF solution (compared to 3) and retained good metabolic stability (Table 3). Compound 9 also demonstrated a lower affinity against a small panel of selected human kinases in addition to Haspin (CDK7, FLT3, KIT, PIM-1,2,3), suggesting that the 2-O-aryl substituent reduces overall human kinase promiscuity.

During this SAR study at the 5-position of the ITDs, an X-ray cocystal structure of the 2,4-difluorobenzene compound 5 with Haspin was solved with a resolution of 1.8 Å (Figure 3). Interestingly, the ITD core is bound to the hinge region via one H-bond between the 7-nitrogen atom and G608 (2.9 Å) and a σ–hole interaction of sulfur with G609 (3.4 Å). The costructure also shows a clear salt bridge interaction between D611 (2.8 Å) of the lower hinge and the ammonium cation of 5. This binding mode of the ITD core projects the 2,4-difluorobenzene ring in a coplanar fashion which binds to Haspin in a hydrophobic pocket with close contact (<3.5 Å) of nearby residues and sheds light on our observed SAR of the 2-OMe or 3-OMe aryl analogs. This hydrophobic pocket likely causes a steric clash with 2-OMe or 3-OMe aryl ITD analogs, mitigating affinity to and inhibition of Haspin kinase while still allowing potent antiplasmodium activity (9 and 12). This theory for antiplasmodium vs human kinase selectivity is further exemplified by the good potency of 4-OMe analogs in both Plasmodium and Haspin assays (10), where there appears to be more room in the Haspin binding pocket. To further probe this binding mode, we introduced a methyl group directly to the 6-position of the ITD core to obtain 33, which led to a significant loss of both antiplasmodium and Haspin activities. Similarly, additional minor modifications of the ITD core resulted in significant loss of antiplasmodium potency, for example, the replacement of the nitrogen with carbon at the 3-position (i.e., imidazothiazole) 34 (Figure 4).

#### Optimization of the 2-Amino-Alkyl Side Chain of ITDs.

Although 9 addressed the issues of the chemical stability and kinase promiscuity, we saw opportunities to further improve upon the potential cardiotoxicity risk (hERG Qpatch IC$_{50}$ 2.4 μM) and absorption, distribution, metabolism, excretion, and toxicity properties. Consequently, the next phase of optimization focused on modifications to the 2-
Table 2. SAR of the 5-Position of ITDs

| cpd | R          | Pf3D7 EC_{50} (μM) | Haspin IC_{50} (μM) | cpd | R          | Pf3D7 EC_{50} (μM) | Haspin IC_{50} (μM) |
|-----|------------|--------------------|---------------------|-----|------------|--------------------|---------------------|
| 3   | -          | 0.025              | 0.011               | 19  |            | 0.13               | -                   |
| 4   | -          | 0.021              | 0.005               | 20  |            | 0.014              | 0.69 (n=2)          |
| 5   |            | 0.009              | 0.003               | 21  |            | 0.028              | 0.078 (n=2)         |
| 8   |            | 0.022              | 1.28 (n=2)          | 22  |            | 3.17               | -                   |
| 9   |            | 0.017              | 1.74                | 23  |            | 2.33               | -                   |
| 10  |            | 0.045              | 0.032 (n=1)         | 24  |            | 0.40               | -                   |
| 11  |            | 0.22               | -                   | 25  |            | 0.44               | -                   |
| 12  |            | 0.028              | 0.77 (n=2)          | 26  |            | 0.41               | -                   |
| 13  |            | 0.009              | 4.96 (n=2)          | 27  |            | 0.067              | 4.04 (n=2)          |
| 14  |            | 0.013              | 5.31 (n=2)          | 28  |            | 0.2                | -                   |
| 15  |            | 0.10               | -                   | 29  |            | >10                | 0.14 (n=1)          |
| 16  |            | 0.079              | 8.07 (n=1)          | 30  |            | 0.02               | 0.045 (n=2)         |
| 17  |            | 0.017              | 1.33 (n=2)          | 31  |            | 0.34               | -                   |
| 18  |            | 0.052              | 0.20 (n=1)          | 32  |            | 0.15               | -                   |

*EC_{50} and IC_{50} values given as averages (n ≥ 3 unless otherwise noted). Standard control mefloquine has an EC_{50} value of 0.034 μM.*
amino-alkyl side chain of the ITD series with an emphasis on reducing the lipophilicity and pK$_a$ while maintaining antiplasmodium activity. Introduction of a hydroxyl group adjacent to the basic amino group led to several vicinal amino-alcohol substituted piperidine analogs, most of which displayed a decreased hERG inhibition with IC$_{50}$'s > 20 μM. Enantiomers 35 and 36 displayed a slight improvement in cell LipE (lipophilic ligand efficiency, LipE = pEC$_{50}$ − log D$_{7.4}$) with a lower pK$_a$ compared to 9, while there is no enantiomeric preference for antiplasmodium activity. Both 35 and 36 have acceptable liver microsomal stability and exhibited good in vivo oral exposure profiles in rats (Table 4). Further reduction of lipophilicity by removing the 4-methyl group of the piperidine ring led to trans-amino-alcohol enantiomers, 37 and 38, which displayed an improvement in cell LipE while maintaining good metabolic stability and reduced pK$_a$, however lower oral exposure was observed in rats with 37 presumably due to a slight drop in permeability and volume. The corresponding cis-amino-alcohol piperidine isomers, 39 and 40, were slightly less potent on Plasmodium, despite their higher basicity. Transposition of the amino-alcohol provided another enantiomeric pair of cis-amino-alcohol analogs, 41 and 42, which showed similar antimalarial activity (EC$_{50}$ = 0.029 μM) with lower basicity (pK$_a$ 7.8), however both enantiomers increased the hERG inhibition (IC$_{50}$ < 20 μM) and 41 had low oral exposure in rats despite its good metabolic stability. The corresponding trans-amino-alcohol analogs, 43 and 44, further reduced the pK$_a$ to 7.5, however the Plasmodium potency was also slightly diminished (EC$_{50}$’s = >0.030 μM). Replacement of the hydroxyl group in 39 and 40 with fluorine led to cis-4-amino-3-fluoro-piperidine isomers 45 and 46, which decreased cellular LipE and increased the hERG inhibition. The corresponding trans-4-amino-3-fluoro-piperidine analog 47 further diminished the Plasmodium potency. Ring contraction led to cis-4-amino-3-hydroxy-pyrroline and 3-amino-3-hydroxymethyl pyrroline analogs as enantiomeric pairs (48 and 49, 50 and 51). While these pyrroline analogs had

### Table 3. Summary of Key Data for 9 for Comparison to 3

|            | 3               | 9               |
|------------|-----------------|-----------------|
| MW, tPSA,  | 323.4, 66.0     | 361.4, 66.5     |
| cLogP, pK$_a$ | 2.91, 9.3      | 1.76, 8.9       |
| Pf3D7 EC$_{50}$ (μM) | 0.025 | 0.017 |
| HepG2 CC$_{50}$ (μM) | 18 | 31 |
| Kinase Kds CDK7/FLT3/Haspin/KIT/PIM1/PIM2/PIM3 (μM) | 0.2/0.1/0.1/0.1 | 12.0/0.6/0.6/0.6/0.6 |
| % chemical degradation (in FaSSIF at 37 °C for 72 h) | 7.8 % (malate salt) | 0.9 % (formate salt) |
| H/R/M Liver Microsome CL (µL/min/mg) | <25/32/<25 | <25/31/38 |
| hERG Qpatch IC$_{50}$ (μM) | 22 | 2.4 |

**Figure 3.** X-ray cocrystal structure of 5 (green) with Haspin kinase. PDB code is 7SQM.

**Figure 4.** Key SAR of ITD-analogs on HASPIN kinase and Pf.
Table 4. SAR of the 2-Amino-alkyl Group

| Cpd | R       | IC50 EC50 (μM) | Quench hERG (μM) | logD pH 7.4 | LipI (cell) | pKa | Rat CLint (nM) @10 mg/kg | Rat AUC (nM%) @10 mg/kg |
|------|---------|----------------|------------------|-------------|------------|-----|------------------------|------------------------|
| 9    | *N-      | 0.017          | 2.4              | 2.0         | 5.8        | 8.9 | -                      | -                      |
| 35   | Me      | 0.02           | >30              | 1.8         | 5.9        | 8.4 | <25 / 28               | 2401                   | 37,977                 |
| 36   | Me      | 0.02           | >30              | 1.7         | 6.0        | 8.3 | <25 / <25             | 1,759                  | 33,773                 |
| 37   | Me      | 0.021          | 21.3             | 1.5         | 6.2        | 8.3 | <25 / <25             | 402                    | 3,956                  |
| 38   | Me      | 0.02           | >30              | 1.3         | 6.4        | 8.3 | <25 / <25             | -                      | -                      |
| 39   | Me      | 0.046          | >30              | 0.9         | 6.4        | 8.7 | -                      | -                      | -                      |
| 40   | Me      | 0.029          | >30              | 0.9         | 6.6        | 8.6 | -                      | -                      | -                      |
| 41   | OH      | 0.029          | 14.4             | 2.1         | 5.4        | 7.8 | <25 / 26.9           | 669                    | 8,254                  |
| 42   | OH      | 0.029          | 9.2              | 2.0         | 5.5        | 7.8 | <25 / 51.7           | -                      | -                      |
| 43   | OH      | 0.031          | 28.5             | 1.6         | 5.9        | 7.5 | -                      | -                      | -                      |
| 44   | OH      | 0.038          | >30              | 1.7         | 5.7        | 7.5 | -                      | -                      | -                      |
| 45   | OH      | 0.028          | 8.8              | 2.1         | 5.5        | 7.8 | -                      | -                      | -                      |
| 46   | F       | 0.036 (n=2)    | 13.9             | 2.3         | 5.3        | 7.8 | -                      | -                      | -                      |
| 47   | F       | 0.082          | -                | 2.4         | 4.7        | 7.2 | -                      | -                      | -                      |
| 48   | NH2     | 0.062          | >30              | 1.6         | 5.6        | 7.4 | -                      | -                      | -                      |
| 49   | OH      | 0.067          | >30              | 1.7         | 5.5        | 7.4 | -                      | -                      | -                      |
| 50   | OH      | 0.066          | >30              | 1.7         | 5.5        | 7.3 | -                      | -                      | -                      |
| 51   | NH2     | 0.045          | 28.3             | 1.6         | 5.7        | 7.3 | -                      | -                      | -                      |
| 52   | OH      | 0.027          | 17.8             | 1.3         | 5.3        | 8.5 | <25 / <25             | 762                    | 8,987                  |
| 53   | OH      | 0.024          | 11.9             | 1.1         | 6.5        | 8.4 | <25 / <25             | -                      | -                      |
| 54   | OH      | 0.056          | >30              | 1.2         | 6.1        | 8.0 | -                      | -                      | -                      |
| 55   | OH      | 0.021          | 18.5             | 1.4         | 6.3        | 8.8 | <25 / 50.1           | 2,260                  | 41,767                 |
| 56   | OH      | 0.017          | 17.7             | 1.9         | 5.9        | 8.2 | <25 / 25.1           | 1,367                  | 20,198                 |
| 57   | OH      | 0.49           | -                | 3.2         | 3.1        | -   | -                      | -                      | -                      |
| 58   | NH2     | 0.130          | -                | 1.5         | 5.4        | 7.3 | -                      | -                      | -                      |
| 59   | SO3Me   | 0.31           | 2.2              | 4.3         | 6.9        | -   | -                      | -                      | -                      |
reduced basicity (pKₐ < 8), they also showed lower Plasmodium potency (EC₅₀ s > 0.045 μM). Transposition of the –OH and –NH₂ in 50 and 51 led to 3-hydroxy-3-aminomethyl pyrrolidine analogs, 52 and 53, which increased the antiplasmodium activity (albeit with higher pKₐ > 8), however oral exposure of 52 was not comparable to the 3,4-hydroxy-aminopiperidine analogs 35 and 36.

To minimize synthetic complexity and avoid the chirality of the 3,4-hydroxy-amino piperidines and pyrrolidines, symmetrical cyclic hydroxy amines were examined. The 3-hydroxy-3-aminomethyl-azetidine analog 54 had diminished Plasmodium potency, however the 4-hydroxy-4-aminomethyl-piperidine analog 55 displayed equipotency with an improved cell LipE, as compared to 35 and 36. Transposition of the hydroxyl and amino groups of 55 led to a lower pKₐ while maintaining good potency and high cell LipE (56). Compound 55 exhibited a comparable oral PK profile in rats to 35 and 36, despite its slightly higher rat microsomal clearance, whereas 56 resulted in a 2-fold lower oral exposure in rats than 55. Replacement of the basic amine in 55 with a second hydroxyl group led to a significant drop in the potency (57; EC₅₀ = 0.49 μM), further confirming the observed SAR and hypothesis that a basic amine is required to obtain potent antiplasmodium activity.

To probe the limits of LipE and further reduce the lipophilicity of 55, the hydroxy group was replaced with a carboxamide and methylsulfone to give 58 and 59, which were significantly less potent on Plasmodium and resulted in lower cell LipE. Homologation of 59 with a methylene carbon improved the antiplasmodium activity, but 60 was still less potent with a lower cell LipE than 55. Replacement of the methylsulfone in 60 with a dimethylsulfonamide did not improve the potency (61). Finally, two cyclic analogs of 55 and 56 were prepared but displayed lower Plasmodium potency and lower cell LipE (62 and 63). Therefore, on the basis of high cellular LipE, low inhibition of hERG, good oral exposure, and low synthetic complexity (lack of the chirality), the 4-(aminomethyl)piperidin-4-ol group (55) was prioritized as an optimal amino-alkyl side chain for the ITD core.

Optimization of LipE and PK/PD Properties: Identification of Clinical Candidate INE963 (1). At this stage of the optimization, our attention turned back to the 5-aryl group, with the aim of further increasing the antiplasmodium potency as well oral exposure, both of which are viewed as critical properties to improve the potential for a single dose treatment. It was hypothesized that increasing lipophilicity would lead to increased tissue binding and volume of distribution (Vₐu), thereby increasing the half-life (T½) and improving in vivo efficacy. However, increasing lipophilicity often adversely affects metabolic stability and off-target selectivity. Therefore, our ambition was to find the most suitable substitution of the 5-aryl-group to combine with the 4-(aminomethyl)piperidin-4-ol side chain and provide a compound with balanced polarity and ideal drug-like properties. To assess the optimal properties for in vivo efficacy, human and rat hepatocyte clearance as well as oral exposure in rats were determined, including the ratio of 48 h concentration over Pf EC₅₀ as a pharmacokinetic/pharmacodynamic (PK/PD) surrogate for maintaining a high parasiticidal concentration over one parasite life-cycle.

Homologation of the 2-OEt group to the 2-OMe group (64) slightly improved the potency but led to lower cell LipE and 2-fold lower oral exposure as compared to 55 (Table 5). Interestingly, the C₄₈ h/EC₅₀ was slightly improved (10.8 vs 9.1) despite the lower exposure. Replacement of the para-fluoro with a methyl group provided 65, which displayed a hERG IC₅₀ of >30 μM with comparable Plasmodium potency to 55. Moreover, 65 had a similar oral exposure with 2-fold higher C₄₈ h/EC₅₀ ratio (18.6 vs 9.1) than 55. It was then hypothesized that introduction of a nitrogen in the ring at the meta-position (adjacent to both ortho-OEt and para-Me) would mitigate lipophilicity and metabolism, while still being shielded from intermolecular interactions. We were subsequently pleased to see that 66 achieved a high cell LipE (6.4) due to improved Plasmodium potency and lower log D, albeit with lower oral exposure and C₄₈ h/EC₅₀ ratio (10.9 vs 18.6) compared to 65. Further systematic exploration of SARs at the para-position revealed that only lipophilic groups were tolerated and identified an isopropyl group, INE963 (1), which improved antiplasmodium activity to single digit nanomolar (EC₅₀ = 0.006 μM). The para-isopropyl group increased overall compound lipophilicity, albeit without significantly increasing in vitro hepatic clearance. Due to these ADME properties, INE963 (1) attained a longer T½ (19.2 h) with a very high C₄₈ h/EC₅₀ ratio (33.3) compared to other analogs. The related 6-isopropyl-2-OEt-pyridyl analog, 67, had similar antiplasmodium potency and exposure in rats, although with lower LipE and increased hERG inhibition (IC₅₀ = 12 μM). Therefore, it was determined that the additional lipophilicity of the ortho-OEt group in 67 provided minimal advantage compared to INE963 (1). For these reasons, and with a focus on selecting a candidate with good potential for single dose treatment in humans, it was determined that

Table 4. continued

| Cpd | R | Ky/D7 (μM) | Quach hERG (IC₅₀ (μM)) | logD pH 7.4 | LipE (cell) | pKa | H/R LM CL₅₀ (μl/min/mg) | Rat C₄₈ h (nM) | Rat AUC (nM h%) |
|-----|---|------------|------------------------|------------|-------------|----|------------------------|---------------|---------------|
| 60  | - | 0.044      | -                      | 2.2        | 5.6         | -  | -                      | -             | -             |
| 61  | - | 0.053      | -                      | 2.2        | 5.1         | 8.0| -                      | -             | -             |
| 62  | - | 0.022      | -                      | 2.7        | 5.0         | -  | -                      | -             | -             |
| 63  | - | 0.062      | -                      | 2.7        | 4.5         | 7.1| -                      | -             | -             |

*Peak-1: Fast-eluting isomer from chiral HPLC. Peak-2: Slow-eluting isomer from chiral HPLC. HLM: Human liver microsomes. RLM: Rat liver microsomes. C₄₈ h, AUC: mean blood concentration time profile from oral administration to rats at 10 mg/kg.
Table 5. SAR of the 5-Aryl Group in Combination with the 4-Hydroxy-4-aminomethyl-piperidine Side Chain

| Cpd | R            | PF3D7 EC50 (nM) | Quatch EREG IC50 (nM) | logD pH 7.4 | LipE (cell) | Hep CLmax (H/R) (μL/min/10^6 cells) | PPB (%) (H/R) | Cmax (nM) | AUC (nM*h) | T1/2 (h) | Cmax/EC50 |
|-----|--------------|----------------|-----------------------|-------------|-------------|-------------------------------------|---------------|----------|-----------|----------|-----------|
| 55  |              | 0.021          | 18.5                  | 1.4         | 5.3         | <4 / 5.5                            | 87.5 / 81.8   | 2.268    | 41,767    | 12.7     | 9.1       |
| 64  |              | 0.014          | 21.5                  | 2.1         | 5.8         | 9.4 / <4                           | 96.8 / 93.5   | 1.207    | 34,433    | 15.7     | 10.8      |
| 65  |              | 0.017          | >30                    | 1.8         | 6.0         | 8.4 / 4.9                           | 81.5 / 87.0   | 2.382    | 48,841    | 14.7     | 18.6      |
| 66  |              | 0.011          | 27.5                  | 1.5         | 5.4         | 9.3 / <4                           | 80.4 / 88.6   | 828      | 17,771    | 15.8     | 10.9      |
| INE963 (1) |              | 0.006          | 23.0                  | 3.1         | 5.2         | <4 / 7.1                           | >99 / >99     | 996      | 31,592    | 19.2     | 33.3      |
| 67  |              | 0.005          | 12.3                  | 3.4         | 4.9         | <4 / <4                           | >99 / >99     | 1,113    | 22,719    | 18.1     | 38.8      |

"Hep CLmax: In vitro hepatocyte clearance. PPB: Plasma protein binding. Cmax, AUC, T1/2: from mean blood concentration time profile of oral administration in rats at 10 mg/kg.

INE963 (1) had the best overall profile and was progressed to further biological characterization.

In Vitro Efficacy and Selectivity. INE963 (1) was profiled in numerous in vitro Plasmodium blood-stage growth inhibition assays and shows excellent potency (Table 6). The EC50 values of INE963 (1) are single digit nanomolar against Pf 3D7 blood stages: EC50 = 3.0–6.0 nM. INE963 (1) is also potent against P. falciparum and P. vivax clinical isolates from Brazil and Uganda with EC50’s ranging from 0.01 to 7.0 nM.19,20 Additionally, INE963 (1) is active against >15 drug-resistant Pf cell lines with EC50’s = 0.5–15 nM. The biological target in parasites has not been identified to date, but investigations are ongoing. The mechanism of action (MoA) appears to be novel due to the activity against multidrug-resistant Pf parasite lines in addition to showing a high barrier to resistance. To date, we have been unable to generate any drug-resistant mutants in selection studies using multiple protocols conducted with various Pf blood stage cultures in vitro. The in vitro selectivity of INE963 (1) for Plasmodium vs human cells was exemplified by approximately 1000+ fold shifts against multiple human kinase biochemical assays as well as respectable margins in multiple cell lines used for cytotoxicity assays (Table 6).

The fast-killing potential of INE963 (1) has been demonstrated in a Pf viability assay (GSK, Tres Cantos) using a limiting dilution technique to quantify the number of Pf 3D7 parasites that remain viable after drug treatment.21,22 The results show no viable parasites after 24 h (5 log reduction) which is comparable to or faster than artesinin. The in vitro log parasite reduction ratio for INE963 (1) is >8.0 at 10 × Pf 3D7 EC50 without a lag phase and a parasite clearance time (PCT99.9) of <24 h (Table 7, Figure 5).

To assess the human kinase selectivity of INE963 (1), the binding affinities to 468 human kinases were measured at high concentration (10 μM) and directly compared with 3, the ITD lead compound. INE963 (1) is significantly more selective (only 10 out of 468 kinase were inhibited at <10% of control)
with a S(10) selectivity score of 0.025. This demonstrates an improved human kinase selectivity compared to 3 (which inhibited 53 out of 468 human kinases at <10% of control) with a S(10) selectivity score of 0.132 (Figure 6). Additionally, the IC_{50}'s of INE963 (1) on Haspin, FLT3, PIK3CA, and PIM1 were measured in a dose response to be 5.51, 3.60, >50.0, and >50.0 μM, respectively, which illustrates a 600–8333-fold selectivity for *Plasmodium* in cell culture. Compared to compound 3 and earlier imidazothiadiazoles from the patent literatures, these data suggest INE963 (1) has been substantially optimized for potency on *Plasmodium* while expressly mitigating binding to human kinases.

**In Vivo Efficacy of INE963 (1).** Based on the potent *in vitro* activity, promising physicochemical properties, and *in vivo* pharmacokinetics, the frontrunner compounds were profiled *in vivo* (Table 8). Oral therapeutic efficacy was evaluated against erythrocytic asexual stages of *Pf* 3D7 *in vivo* in a humanized severe combined immunodeficient (SCID) mouse model measuring the effect on blood parasitemia by flow cytometry and microscopic analysis. Efficacy was assessed following oral administration of the compounds either as four doses or as one single oral dose, and the effect on blood parasitemia was measured up to 60 days post-treatment.

Table 7. *In Vitro P. falciparum* Kill Curve Parameters of INE963 (1)  

| cpd     | dose lag phase (h) | log PRR | PCT 99.9% (h) |  |
|---------|--------------------|---------|---------------|---|
| INE963 (1) | 10 × EC_{50}     | >8.0    | <24            |  |
| artemisinin | 10 × EC_{50}     | >8.0    | <24            |  |

"Estimation based on the first 24 h of treatment.

Table 8. *In Vivo* Efficacy of ITD Lead Compounds in *Pf* 3D7 Humanized SCID Mouse Malaria Model  

| parameter | 55 | 60 | 65 | INE963 (1) |  |
|-----------|----|----|----|------------|---|
| dose\(a\) | 4  | 30 | 4  | 30         |  |
| activity\(b\) | >99.9 | >99.9 | >99.9 | >99.9 |   |
| DoR\(c\) | -  | >60 | >60 | >60 |   |
| C\(_{max}\) | 1.2 | 0.59 | 1.2 | 0.59 |   |
| AUCC_{23h}\(d\) | 15.5 | 4.5 | 8.5 | 5.1 |   |
| AUCC_{47h}\(d\) | -   | -   | -   | -   |   |

| dose\(a\) | 1  | 5  | 1  | 50 | 1 | 3 | 1 | 10 |
| activity\(b\) | >99.9 | >99.9 | >99.9 | >99.9 |   |
| DoR\(c\) | 13.8 | 16.8 | >60 | >60 |   |
| C\(_{max}\) | 0.16 | 0.40 | 0.80 | 1.5 |   |
| AUCC_{23h}\(d\) | 1.3 | 3.5 | 6.9 | 16.7 |   |
| AUCC_{47h}\(d\) | 1.7 | 4.5 | 8.2 | 29.8 |   |

\(a\) Data from multiple experiments; n = 1 for four dose groups; n = 2 for compound 3; n = 1 for 3, 10, 30, and 100 mg/kg of INE963 (1); n = 3 for 15 and 20 mg/kg of INE963 (1). Average values are shown wherever there is more than one animal. \(b\)Dose in mg/kg. Activity = Parasitemia reduction compared to untreated controls, in %. \(c\)DoR = Day of recrudescence, in day. \(d\)C\(_{max}\) = Maximum concentration, in μg/mL. AUCC_{0-23 h} = Area under the curve from time 0–23 h postdose, in μg*h/mL. AUCC_{0-47 h} = Area under the curve from time 0–47 h postdose, in μg*h/mL.

Following four oral doses at 30 mg/kg, the frontrunner compounds tested showed significant parasitemia reduction (>99.9%) at day 5 compared to untreated control mice. In addition, the animals did not show any signs of recrudescence of parasite at day 60 post-treatment, indicating complete cure at these doses. Following on these promising results, INE963 (1) was further evaluated in single oral doses. In mice treated with a single oral dose of 15 mg/kg and above, INE963 (1) showed >99.9% parasitemia reduction at day 5 postdosing compared to untreated control mice. The effect of a single oral dose was rapidly cidal and reached maximum killing at 15 mg/kg. The effective dose to reduce 90% parasitemia (ED_{90}) was determined to be 11.7 mg/kg. However, with a single dose of 3 mg/kg, 10 mg/kg, 15 mg/kg and 20 mg/kg p.o., mice showed recrudescence of parasites on day 0, day 5, day 14, and day 17, respectively, whereas with a single dose of 30 and 100 mg/kg p.o., mice did not show any signs of recrudescence of parasites at day 60 post-treatment, indicating a complete cure at these doses (Table 8, Figure 7A). Furthermore, dose-related increases in C_{max} and AUCC were also observed. The threshold exposure (AUCC_{0-47h}) in this preclinical Pf SCID mouse model required for 99.9% parasitemia reduction was 2.1 μg*h/mL (Figure 7B) and for complete cure (>60 days) was 8.2 μg*h/mL (Figure 7C). The results from this study, in conjunction with the predictions of human PK, demonstrate INE963 (1) has the potential for single-dose cures of uncomplicated malaria.
**Pharmacokinetic Properties of INE963 (1).** INE963 (1) has high in vitro permeability of $9.9 \times 10^{-6}$ cm/s based on the low efflux Madin Darby canine kidney cell line (MDCK-LE) assay. INE963 (1) is expected to be a P-gp or multidrug resistance 1 (MDR1) protein substrate based on an efflux ratio of 11.8 using transfected MDR1-MDCK cells. However, due to high permeability, this did not significantly impact oral bioavailability. The free-base INE963 (1) is highly crystalline as observed by X-ray powder diffraction (XRPD), and while this form has very low solubility in water (0.0002 mg/mL), there is a dramatic increase of the solubility in SGF (pH 7.4) >2 and in FaSSIF pH 6.5 (1.3 mg/mL) which likely has a positive impact on oral absorption. Overall, INE963 (1) is a lipophilic base, $\log D_{2,4} = 3.1$ and $pK_a = 8.7$, with high solubility and is classified as a BCS class III drug. INE963 (1) displays minimal inhibition of CYP450s, with measured IC_{50}'s ranging from 4.5 to 8.5 μM for 2C9, 2C19, 2B6, and 2C8; with all others >20 μM. Across the species tested, plasma protein binding is >99% with low in vitro microsomal and hepatocyte clearance (CL_{int}). In addition, INE963 (1) exhibits high membrane and tissue binding in vivo (i.e., high volume) which minimizes the amount of free drug exposed to hepatic clearance and thus increases the apparent in vivo $T_{1/2}$ (Table 9).

In vivo pharmacokinetics of INE963 (1) have been studied in mice, rats, and dogs where the absorption was moderate to high ($T_{max} = 1-24$ h) with good bioavailability (%F) of 39–74% across the species. INE963 (1) has a low blood clearance (<10% of hepatic blood flow in rat and mouse and <20% of hepatic blood flow in dog) and a high volume of distribution, resulting in long half-lives (15–24 h) across the species (Table 9).

**In Vitro Metabolism of INE963 (1).** The in vitro metabolism of INE963 (1) was first investigated following incubation in rat, dog, and human cryopreserved hepatocytes at 10 μM for up to 4 h, resulting in two metabolites: hydroxylation on the isopropyl moiety (<2.5%) with subsequent dehydration (<3.5%). Neither uptake issues (MDCK-LE permeability = $9.9 \times 10^{-6}$ cm/s) nor saturation of metabolic enzymes were responsible for the low metabolic turnover of INE963 (1), as high metabolic stability was also observed in human liver microsomes at 0.5 and 6 μM. Long-term in vitro incubations may increase metabolite formation for low turnover compounds.25,26 Subsequently, INE963 (1) was incubated for up to 168 h in rat, dog, and human primary hepatocyte/nonparenchymal stromal cell cocultures (at 10 μM). A summary of the in vitro metabolites obtained from this study is provided in Figure 8. Hydroxylation and demethylation represented the main phase I metabolic pathways, whereas acetylation (rat and human) and formylation (mainly in dog) seemed to be the main phase II metabolic pathways in vitro. Regardless of the species, hydroxylated INE963 (M13) was the most intense in vitro metabolite based on mass spectrometric responses. Moreover, all in vitro metabolites

### Table 9. Summary of Physiochemical and Pharmacokinetic Properties of INE963 (1)

| assay | INE963 (1) data |
|-------|----------------|
| physical form by XRPD | free base; highly crystalline |
| $pK_a$ | 4.0, 8.7 |
| $\log D_{2,4}$ | 3.1 |
| solubility in water (mg/mL) | 0.0002 |
| solubility in FaSSIF pH 6.5 (mg/mL) | 1.3 |
| solubility SGF pH 2.0 (mg/mL) | >2 |
| permeability (MDCK-LE; Papp AB × 10^6 cm/s) | 9.9 |
| CYP inhibition (2C9, 2C19, 2B6, 2C8, all others >20 μM) | 4.5, 5.4, 6.0, 8.5 |
| efflux ratio (MDR1-MDCK; BA/AB) | 11.8 |
| biopharmaceutical class (BCS class) | III (up to 1000 mg dose) |
| mouse, rat, dog, human PPB (% bound) | >99, >99, >99, >99 |
| mouse, rat, dog, human microsome CL_{int} (μL/min/mg) | <25, 29, <25, 25.4 |
| rat, dog, human hepatocyte CL_{int} (μL/min/10^6 cells) | 7.1, <4, <4 |
| mouse, rat, dog in vivo CL (μL/min/kg)^a | 4.0, 5.9, 5.4 |
| mouse, rat, dog in vivo V_a (L/kg)^a | 7.0, 8.3, 6.3 |
| mouse, rat, dog in vivo half-life $T_{1/2}$ (h)^a | 22.5, 20.4, 15.1 |
| mouse, rat, dog in vivo oral bioavailability (%F)^a | 47, 39, 74 |

"In vivo PK data from a crystalline formate-salt batch of INE963 (1), using blood concentration.

![Figure 7. Parasitemia dose–response relationship of INE963 (1) in P f 3D7 humanized SCID mouse model following one single oral dose (A) and the exposure (AUC_{0–48h}) response relationship with respect to parasitemia reduction (B) and day of recrudescence (C). Data are from multiple experiments with n = 2 for vehicle treatment in each experiment, n = 1 for 3, 10, 30, and 100 mg/kg; n = 3 for 15 and 20 mg/kg dose groups.](image-url)

![Figure 8. In vitro metabolism of INE963 (1) following incubation in rat, dog, and human primary hepatocyte/nonparenchymal stromal cell cocultures at 10 μM for up to 168 h.](image-url)
formed in human hepatocytes were also obtained following incubation in rat or dog hepatocytes. Current investigations are ongoing to further assess the metabolism of INE963 (1) in vivo, which will be published at later stage.

Human Pharmacokinetic Prediction of INE963 (1). Due to the low or no hepatic turnover in vitro, either liver microsomal or hepatocyte assays under-predicted the in vivo clearance observed in preclinical species. For this reason, a direct in vitro vs in vivo extrapolation could not be established. Allometry-based approaches using in vivo PK data from mice, rats, and dogs were utilized to predict human clearance (CL) and steady-state volume (Vss). Since no human-specific metabolites have been observed from in vitro studies and INE963 (1) has low CL and high Vss across all species in vivo, the overall confidence in using allometry methods is good. Using all three species data, clearance and steady-state volume of distribution in humans were estimated as 1.6 mL/min/kg, and 7.2 L/kg, respectively. From these parameters, the Wajima method was used to generate a predicted human IV time-concentration plot, and further a gut PBPK model was used for the prediction of a human oral PK profile. The predicted human PK parameters are summarized in Table 10. The bioavailability is estimated to be ~70% after a single oral dose as an immediate release (IR) tablet under fed conditions, and the apparent T1/2 is predicted to be long (~60 h). Overall, the forecasted PK profile is supportive of an oral single-dose curative therapy in patients. Based on emerging PK data from the first-in-human study, modeling is in progress to identify single doses sufficient to maintain efficacious concentrations (estimated to be from 35 to 150 nM based on the SCID mouse model) for multiple Plasmodium blood-stage life cycles (4–6 days).

### CONCLUSION

In summary, INE963 (1) offers several advantageous properties as an antimalarial candidate for single-dose cure therapy of uncomplicated malaria, including moderate-to-high bioavailability with a projected human half-life of ~60 h. In the humanized SCID mouse Pf malaria model, INE963 (1) achieved 99.9% parasite reduction in blood with a single oral dose of 15 mg/kg and a >60 days full cure with a single oral dose of 30 mg/kg. Furthermore, INE963 (1) shows high potency on resistant strains and field isolates, a high barrier to resistance in vitro, a fast-acting phenotype in Plasmodium assays, and a MoA that is unknown but likely to be novel. INE963 (1) also has a high degree of selectivity (>1000-fold) against comprehensive in vitro safety panels, including human kinases that have previously been reported as sensitive to 5-aryl-2-amino-ITDs. Additionally, INE963 (1) was evaluated in off-target safety panels, GLP safety pharmacology, genetic toxicity, and 2-week GLP toxicity studies in rats and dogs and was determined to have an adequate safety profile for advancement into clinical studies. Taken together, the balance of these properties position INE963 (1) as a unique antimalarial clinical candidate with a high barrier to resistance and the potential for single-dose cures. Phase 1 clinical studies are currently underway in healthy volunteers.

#### SYNTHESES

The gram-scale synthesis of INE963 (1) outlined in Scheme 1 commenced with bromination of 1,3,4-thiadiazol-2-amine (68), which then underwent cyclization with 2-chloroacetaldehyde, followed by iodination to furnish key intermediate 2-bromo-5-iodo-imidazothiadiazole (71). The Corey–Chaykovsky reaction of 1-benzylpyridin-4-one (72) provided the epoxide 73 in high yield, which was subsequently opened up with ammonia before Boc-protection to yield 75. Removal of the benzyl group furnished intermediate 76 which underwent an SnAr reaction with the bromide of key intermediate 71 to yield 77. The boronic ester 80 was prepared in two high yielding steps starting with an iron-catalyzed addition of isopropyl Grignard reagent to 2-chloro-6-methoxypyridine (78), followed by treatment with n-butyllithium and subsequent reaction with tri-isopropyl borate to yield 80. Suzuki coupling of 80 with 77, followed by Boc group removal with formic acid, then provided INE963 (1).

Since the route described in Scheme 1 offered substantial flexibility and was amenable to gram-scale synthesis of INE963 (1), we implemented a general synthetic approach shown in Scheme 2.

### Table 10. Summary of Predicted Human Pharmacokinetic Parameters for INE963 (1)

| PK parameter | predicted human value | units | notes |
|--------------|-----------------------|-------|-------|
| clearance (CL) | 1.6 | mL/min/kg | based on allometric scaling method with brain weight correction |
| volume steady state (Vss) | 7.2 | L/kg | mean values from allometric scaling using single species (mouse, rat, dog) |
| apparent T1/2 | ~60 | hours | fit to 3-compartmental model with linear elimination after IV dose (GastroPlus) |
| bioavailability (F) | ~70 | % | a gut PBPK model with an ACAT model after an oral dose at 1 mg/kg in IR tablet (Fed) (GastroPlus) |
starting with the key intermediate ITD 71. A facile SnAr reaction with a variety of primary and secondary amines furnished the 2-amino-substituted imidazothiadiazole 82 in a moderate to high yield. Subsequently, cross-coupling with a range of different boronic acids/esters using palladium catalysis followed by Boc deprotection gave the target compounds 83.

**EXPERIMENTAL SECTION**

**General Procedures.** All materials and reagents used were of the best commercially available grade and used without further purification. Normal-phase column chromatography was carried out using prepacked silica gel cartridges on a CombiFlash system by Teledyne ISCO.1H NMR spectra were determined on a Bruker DRX-400 spectrometer in deuterated solvents. Mass spectra were recorded using a Q-TOF high-resolution quadrupole time-of-flight mass spectrometer. Preparative HPLC was performed on a Waters Prep HPLC system using a C18 reversed-phase column eluting with gradient mixtures of water/acetonitrile containing a modifier 0.05% trifluoroacetic acid. All compounds >95% purity by HPLC analysis. The purity data have been confirmed by 1H NMR, LCMS, and HRAMS in the Supporting Information. The human biological samples were sourced ethically, and their research use was in accord with the terms of the informed consents under an IRB/EC approved protocol. All animal studies were ethically reviewed and carried out in accordance with European Directive 2010/63/EEC and the GSK Policy on the Care, Welfare, and Treatment of Animals. Human erythrocytes for all animal studies were obtained from the Basque Center of Transfusion and Human Tissues, Galdakao, Spain; the Centro de Transfusiones de la Comunidad de Castilla y León, Valladolid, Spain; and the Bank of Blood and Tissues, Barcelona, Spain. Clinical isolate assays in Brazil and Uganda were performed as previously described.

**5-Bromo-1,3,4-thiadiazol-2-amine (69).** To 1,3,4-thiadiazol-2-amine (68) (500 g, 4.95 mol) in MeOH (5 L), NaHCO3 (830.9 g, 12.26 mol) was added at room temperature (RT). The mixture was cooled to 0 °C, and bromine (792.0 g, 4.95 mol) was added dropwise over a period of 1 h. The mixture was warmed to RT and stirred for 3 h, and the solid was filtered and washed with MeOH (500 mL). The solid was slurried in water (10 L) for 1 h and collected and washed with water (1 L). The solid was slurried in MeOH (1.5 L), filtered, and washed with MTBE (1 L) to give the title compound (788.4 g, 74% yield) as a yellowish liquid. 1H NMR (400 MHz, CDCl3): δ ppm 7.51 (brs, 2H). ESI MS (m/z): [M + H]+ calcd for C18H29N2O3 321.2, found 321.2. 6-Benzyl-1-oxa-6-azaspiro[2.5]octane (73). BuOK (177.9 g, 1.59 mol) was added in portions to DMSO (2.0 L) at RT under N2, then trimethylsilylfluorosiloxane iodide (319.8 g, 1.45 mol) was added in portion slowly over 15 min at RT. The resulting solution was stirred at RT for 1 h. A solution of 1-benzyllpiperidin-4-ol (72) (250 g, 1.32 mol) in DMSO (0.5 L) was added at RT over 30–40 min. The reaction mixture was stirred at RT for 1 h, then the solution was quenched with water (2.5 L) and extracted with MTBE (2.5 L × 2). The combined organic layers were washed with brine (2.5 L), dried over Na2SO4, filtered, and concentrated to give the title compound (253 g, 94%) as yellow solid.

**4-(Aminomethyl)-1-benzylpiperidin-4-ol (74).** To 6-benzyl-1-oxa-6-azaspiro[2.5]octane (73) (250 g, 1.23 mol) in MeOH (1.5 L), aqueous ammonia (3 L, 4.15 mol) was added dropwise at 5–10 °C over 15 min. The resulting mixture was stirred at RT for 16 h and diluted in DCM (3.5 L). The aqueous layer was separated and extracted with DCM (3 L × 3). The combined organic layers were washed with NaOH (1 N, 3 L) and brine (3 L), dried on Na2SO4, and concentrated to give the title compound (225 g, 83%) as off-white solid, which was used for next step without further purification.

**tart-Butyl ((1-Benzyl-4-hydroxyperidin-4-yl)methyl)carbamate (75).** 4-(Aminomethyl)-1-benzylpiperidin-4-ol (74) (430 g, 1.95 mol) was suspended in 2-Me THF (8.6 L) and Et3N (197.5 g, 1.95 mol) was added before the reaction was cooled to 0 °C. A solution of Boc2O (511.5 g, 2.34 mol) in 2-Me THF (400 mL) was added at 0 °C over 20 min. The resulting solution was stirred for 16 h, and the mixture was washed with MeOH (2 L) and brine (2 L). The organic layer was washed with MeOH (2 L × 2), dried over Na2SO4, filtered, and concentrated to give the title compound (396 g, 62%) as white solid.

**2-Bromo-5-iodoimidazo[2,1-b][1,3,4]thiadiazole (70).** To a mixture of 5-bromo-1,3,4-thiadiazol-2-amine (69) (500.0 g, 2.78 mol) in water and EtOH (1:1; 5 L) was added a 44% aqueous solution of 2-chloroacetaldehyde (1425.9 g, 7.78 mol) over a period of 15 min at RT. The reaction mixture was heated at reflux over a period of 1 h and stirred for 48 h. After completion of the reaction, the reaction mixture was quenched with 8% aqueous solution of sodium bicarbonate (5 L), and EtOAc (10 L) was added to it. The mixture was stirred for 10 min at RT. The reaction mixture was filtered through Celite bed, and bed was washed with EtOAc (1.0 L). The organic layer was separated, and an aq. layer was extracted with EtOAc (1.0 L × 2). The combined organic layer was concentrated and purified on silica gel (100–200 mesh, EtOAc in heptane from 16.7% to 25%) to give the title compound (88.5 g, 20%) as yellowish solid. 1H NMR (300 MHz, DMSO-d6): δ ppm 8.22 (d, J = 1.2 Hz, 1H), 7.39 (d, J = 0.8 Hz, 1H). ESI MS (m/z): [M + H]+ calcd for C16H11BrN3S 312.1, found 312.1. 2-Bromo-5-iodoimidazo[2,1-b][1,3,4]thiadiazole (71). To 2-bromimidazo[2,1-b][1,3,4]thiadiazole (70) (50.0 g, 245 mmol) in DMF (500 mL), NIS (66.1 g, 294 mmol) was added at RT on portion, and the resulting mixture was stirred for 2.5 h. Another NIS (110.0 g, 449 mmol) was added and stirred for another 1.5 h. The reaction mixture was diluted with EtOAc (2.0 L), and then the reaction mixture was washed with aqueous Na2SO4 solution (500 mL). The organic layer was washed with ice cooled water (3 × 250 mL) and further washed with brine (250 mL), dried over anhydrous Na2SO4, filtered, and concentrated. The residue was slurried in MeCN (100 mL) for 30 min, and the solid was filtered and washed with MeCN (50 mL) to give the title compound (40.4 g, 37%) as white solid. 1H NMR (400 MHz, DMSO-d6): δ ppm 7.43 (s, 1H). ESI MS (m/z): [M + H]+ calcd for C17H14BrN3S 329.8, found 329.7.

**Scheme 2. General Pathway to Synthesize Imidazothiadiazole Analogs**

Reagents and conditions: (i) RR′NH, DIPEA, CH3CN or NMP or DMSO, 90–110 °C; (ii) R−boronic acid/ester, PdCl2(dppf)-DCM (5 mol %), aq. K3PO4, 1,4-dioxane, 80–90 °C; (iii) HCl or HCOOH or TFA, 0 °C to RT.
tart-Butyl (4-(4-Hydroxy-1-(5-iodoimidazo[2,1-b][1,3,4]thiadiazol-2-yl)piperidin-4-yl)methyl)carbamate (77). A mixture of tert-butyl ((4-hydroxypropidin-4-yl)methyl)carbamate (76) (155 g, 0.76 mol), 2-bromo-5-iodoimidazo[2,1-b][1,3,4]thiadiazole (232.3 g, 0.71 mol), and DIPEA (174.0 g, 1.35 mol) in 2-MeTHF 1.55 L, 10 V) was heated to 85 °C for 9 h. The reaction was stirred in water (10 V) at RT for 15 min before the layers were separated. The aqueous layer was extracted twice with EtOAc (800 mL), dried over Na2SO4, distilled under vacuum at 40 °C to give the title compound (168 g, 52%) as light brown solid. The solids were slurried in MeCN (360 mL), and then dried under vacuum at 45 °C and further diluted with water (800 mL) at RT for 15 min before the layers were separated. The aqueous layer was extracted twice with EtOAc (200 mL) and then dried under vacuum at 40 °C to give the title compound (123 g, 70%) as a white solid.1H NMR (400 MHz, CDCl3): δ ppm 8.50 (d, J = 7.7 Hz, 1H), 7.56 (s, 1H), 7.00 (d, J = 7.8 Hz, 1H), 4.42 (s, 1H), 4.35 (d, J = 10.4, 6.5 Hz, 2H), 3.8−3.41 (m, 2H), 3.33 (s, 2H), 2.97 (h, J = 6.8 Hz, 1H), 1.63−1.56 (m, 4H), 1.26 (d, J = 6.8 Hz, 2H). ESI HREMS [M + H]+ calced for C15H22IN5O3S 480.1, found 480.3. 6-Isopropyl-2-(5-iodoimidazo[2,1-b][1,3,4]thiadiazol-2-yl)piperidin-4-yl)boronic acid (81). To a solution of 2-chloro-6-methoxypyridine (76.08 g, 0.39 mol) in 1,4-dioxane (1.7 L) under N2 atmosphere was added a prepared solution of K3PO4 (225.85 g, 1.064 mol) and DIPEA (174.0 g, 1.35 mol) in 2-MeTHF 1.55 L, 10 V). The combined organic layer was washed with brine, dried over Na2SO4, filtered through Celite bed at 30 °C, and then tri-isopropyl trialkylsilane (5-iodoimidazo[2,1-b][1,3,4]thiadiazol-2-yl)piperidin-4-yl)boronic acid (81) (50 g, 0.099 mol) was collected to 0 °C before formic acid (80 mL, 9.95 mol) was slowly added under N2 atmosphere. The reaction mixture was allowed to warm at RT and continue to stir for 7 h at RT. The excess of formic acid was evaporated under reduced pressure at 30 °C and then distilled with MeCN (5 × 360 mL). The resulting solids were slurried in MeCN (150 mL) and then dried to obtain 37 g of formate salt. Combined with another similar batch size, the formate salt (72 g) was dissolved in water (580 mL) and a solution of NaOH (20% in Water, 72 mL) was added to adjust the pH to 13. The mixture was stirred for 1 h at RT. The solids were filtered and washed with water (360 mL), then washed with MTBE (360 mL), and finally dried under vacuum for 16 h. The solids were taken again in water (720 mL) and stirred for 2 h at RT. The solids were filtered, washed with water (360 mL), washed with MTBE (360 mL), and then dried under vacuum at 45 °C for 48 h to give the title compound (62 g, 72%). 1H NMR (300 MHz, DMSO-d6): δ ppm 8.50 (d, J = 7.7 Hz, 1H), 7.56 (s, 1H), 7.00 (d, J = 7.8 Hz, 1H), 4.42 (s, 1H), 4.35 (d, J = 10.4, 6.5 Hz, 2H), 3.8−3.41 (m, 2H), 3.33 (s, 2H), 2.97 (h, J = 6.8 Hz, 1H), 1.63−1.56 (m, 4H), 1.26 (d, J = 6.8 Hz, 2H). ESI HREMS [M + H]+ calced for C15H22IN5O3S 480.1, found 480.3. 

**Pf 3D7 Phenotypic Assay.** Following an established Pf protocol,27 parasite cultures were grown in media (RPMI 1640 medium (10.4 g/L) with 0.5% Albumax II, 200 μM hypoxanthine, 50 mg/L gentamicin sulfate, 35 mM Hesper, 2.0 g/L sodium bicarbonate, and 11 mM glucose) and human erythrocytes. Cultures were maintained at 37 °C in an incubator with 5% CO2. In vitro antiplasmodium activity was measured according to a modified SYBR Green cell proliferation assay.28 Dose−response curve data were normalized based on fluorescence signal values from DMSO treated wells (0% inhibition) and mefloquine treated wells (100% inhibition) at a final concentration of 10 μM. The standard logistic regression model was applied for curve fitting in order to determine the EC50.20 HespG2 Cytotoxic Assay. HespG2 are adherent cells that were maintained in Dulbecco’s modified Eagle’s medium-F12 supplemented with 1% penicillin/streptomycin and 10% heat-inactivated FBS. Fifty μL of a 5 × 105 cells/mL suspension were dispensed in white, solid 384-well plates (Greiner). Cells were exposed to compounds for 72 h, after which cell viability was quantified by using CellTiter-Glo. This reagent measures ATP release based on the mono-oxidation of luciferin catalyzed by Mg2+, ATP, and molecular oxygen. EC50 values were calculated from two independent experiments performed in triplicate using the Graphpad Prism software. The standard logistic regression model was applied for curve fitting in order to determine the EC50.20 Haspin Kinase assay. The assay was done in assay buffer consisting of 50 mM 4-(2-hydroxymethyl)-1-piperazine ethanesulfonic acid or HEPES at pH 7.5, 5 mM MgCl2, 0.01% Tween-20, 0.1% glycerol, 0.5 mM (tris(2-carboxyethyl)phosphine) or TCEP, and 0.01% bovine serum albumin. All reaction was performed at RT. Compounds were prepossed and incubated with 10 μL of assay buffer containing 0.05 μM of human Haspin kinase domain for 30 min. The reaction was initiated by adding 10 μL of assay buffer containing 0.3 mM H3 biotin peptide and 30 mM of ATP for 90 min. The assay was stopped with 100 μM EDTA. The luminescence signal
was detected by adding using 10 mg/mL of streptavidin donor beads and 42 ng/mL of antiphospho-histone H3 (Thr3) antibody together with 10 mg/mL protein A acceptor beads. The plates were briefly spun, sealed, and incubated in the dark overnight before reading them with Envision.

**PF 3D7 Phenotypic Resistance Selection Methods.** Three different protocols were used to raise resistance against ITD leads in *Pf* blood stages. Culture conditions were as described. Asexual parasites were exposed to a drug concentration of 3 × 10^{-6} IC_{50} at a single inoculum of 10^{6} parasites in duplicates. The parasites cleared from the culture within days. The cultures were maintained and monitored for parasites growth on a routine basis. When no parasites recrudesced within 60 days, the cultures were discarded. The experiment was repeated in the presence of 5 μM ethylmethanesulfonate (EMS), a mutagen that increases the chance for resistance development. 10^{6} parasites were incubated in duplicate for 2 h with EMS prior to applying the drug pressure to raise resistant parasites. Cultures were again monitored for 60 days before discarding them after no recrudescence was observed. Lastly, we exposed parasites to low doses of drug cycling over a period of 7 months. 10^{6} parasites were maintained between 0.5% and 10% parasitemia and exposed to short pulses of drug incubation (1–4 days). After each treatment, the drug was removed, and parasites were allowed to recover. The drug concentration was slowly raised from 0.5 × 8 × IC_{50} but parasites either failed to recover or did not show a resistant phenotype as measured by 72 h growth inhibition assays. The experiment was run in duplicate and four biological replicates.

**In Vivo Efficacy Studies using *P. falciparum* SCID Mouse Model.** In vivo animal experiments were performed at The Art of Discovery SL. These studies were approved by The Art of Discovery Institutional Animal Care and Use Committee (TAD-IACUC). The committee is certified by the Biscay County Government (Biskaiiko Foru Aldundia, Basque Country, Spain) to evaluate animal research projects from Spanish institutions according to point 43.3 from Royal Decree 53/2013, from the first of February (BOE-A-2013-1337). All experiments are carried out in accordance with European Directive 2010/63/EU.

The compound efficacy was assessed in the murine *P. falciparum* SCID model as described previously. Briefly, 22–28 g female NOD-SCID IL-2Rnull mice (NSG) (Charles River, France) previously engrafted with human erythrocytes (>40% of human erythrocytes in peripheral blood) were intravenously infected with *Pf* 3D70087/N9-infected erythrocytes (0.3 mL of 1.17 × 10^{8} parasitized-erythrocytes per mL) 72 h before drug treatment inception. At this point (day 1 of the study), mice had 1–2% of parasitemia on average, and the mice were randomly allocated to selected treatments. Compound was formulated in 0.5% methylcellulose and 0.5% Tween 80 in double distilled water administered by oral gavage at 10 mL/kg dose volume on day 1 of the study either as four oral doses or one single oral dose. The effect of treatment on parasitemia was assessed by measuring the percentage of infected erythrocytes in peripheral blood every 24 h until parasitemia was below the selected limit of quantitation (usually 0.01%) by flow cytometry. The parasitemia in mice was regularly measured up to day 60 of experiment to check the presence of circulating parasitized human erythrocytes. In addition, during the study, samples of peripheral blood were taken between 0 and 71 h postdose from mice to measure the concentration of drugs in blood after oral administration. The drugs were extracted from blood samples by protein precipitation using standard liquid–liquid extraction. Briefly, 10 μL of blood samples were mixed with 60 μL of acetonitrile containing internal standard (100 ng/mL of trimipramine-d8, in methanol), vortexed, and centrifuged, 60 μL of supernatant was mixed with 55 μL of water. These samples were analyzed by LC-MS/MS for quantification in a Waters Micromass UPLC-TQD (Waters, Manchester, UK) under optimized conditions. Data analysis is performed using Phoenix WinNonlin version 7.0 (Certara) to calculate PK parameters via noncompartmental analysis.
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■ ABBREVIATIONS USED

acac, acetylcacetone; ADME, absorption, distribution, metabolism, and excretion; AUC, area under the curve; Boc₂O, di-tert-butyl dicarbonate; CL, clearance; DCM, dichloromethane; EC₅₀, half-maximal effective concentration; ED₅₀, effective dose 50; FaSSIF, fasted state simulated intestinal fluid; ITD, imidazothiadiazole; 2-MeTHF, 2-methyltetrahydrofuran; MMV, Medicines for Malaria Venture; MTBE, methyl tert-butyl ether; NIS, N-iodosuccinimide; NMP, N-methyl-2-pyrrolidone; Pd(dppf)Cl₂, DCM, [1,1’-bis-(diphenyolphosphino)ferrocene] dichloropalladium(II), complex with dichloromethane; Pf, Plasmodium falciparum; PBP, plasma protein binding; RT, room temperature; SAR, structure–activity relationship; SCID, severe combined immunodeficiency; T₁/₂, half-life; TEMDA, tetramethylethlenediamine; Vᵣ, volume steady state

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