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Robenidine Analogues Are Potent Antimalarials in Drug-Resistant *Plasmodium falciparum*

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ABSTRACT: Robenidine is a veterinary drug used in the poultry industry to treat coccidiosis caused by parasites in the *Eimeria* genus. Though this compound and related aminoguanidines have recently been studied in other pathogens, the chemotype has not been systematically explored to optimize antimalarial activity despite the close genetic relationship between *Eimeria* and *Plasmodium* (both are members of the Apicomplexa phylum of unicellular, spore-forming parasites). In this study, a series of aminoguanidine robenidine analogues was prepared and tested *in vitro* against *Plasmodium falciparum*, including multidrug-resistant strains. Selected compounds were further evaluated *in vivo* against murine *Plasmodium yoelii* in mice. Iterative structure–activity relationship studies led to the discovery of **1**, an aminoguanidine with excellent activity against drug-resistant malaria *in vitro* and impressive *in vivo* efficacy with an ED_{50} value of 0.25 mg/kg/day in a standard 4-day test.

Aminoguanidine
Compound 1

KEYWORDS: robenidine, Plasmodium, Eimeria, aminoguanidine, malaria, coccidiosis

Malaria remains a devastating parasitic disease responsible for 229 million infections and 409 000 deaths in 2019.[1] Among the species of malaria parasites that can infect humans, *Plasmodium falciparum* is the most virulent and deadly. Pregnant women and children are the most vulnerable to mortality from malaria, and most cases and deaths occur in sub-Saharan Africa, where *P. falciparum* is the dominant species. Widespread resistance to existing therapies for malaria is an increasingly significant concern for global health. Drug resistance continues to spread for frontline malaria therapies, and resistance has emerged in the past decade in Cambodia and neighboring countries for current "last line of defense" drugs such as artemisinin.[2] The spread of artemisinin resistance outside of southeast Asia seems inevitable and threatens to reverse the recent progress that has been made in decreasing malaria deaths worldwide, i.e., from the year 2000 to 2019, deaths have fallen from 736 000 to 409 000.

Development of new malaria drug classes that can evade existing resistance mechanisms is an urgent global need if elimination and eradication goals are to be achieved.[3] One frequently proven successful approach to the development of novel therapeutics is to begin with a drug for a related parasite or pathogen and refine its structure using medicinal chemistry techniques.

Robenidine (Figure 1a), formerly called robenzidene, is a veterinary drug developed in the early 1970s to treat coccidiosis caused by parasites in the *Eimeria* genus.[4−10] Robenidine is frequently added to animal feed to prevent and treat coccidiosis in chickens,[11] other fowl,[12] and rabbits.[13]

a

b
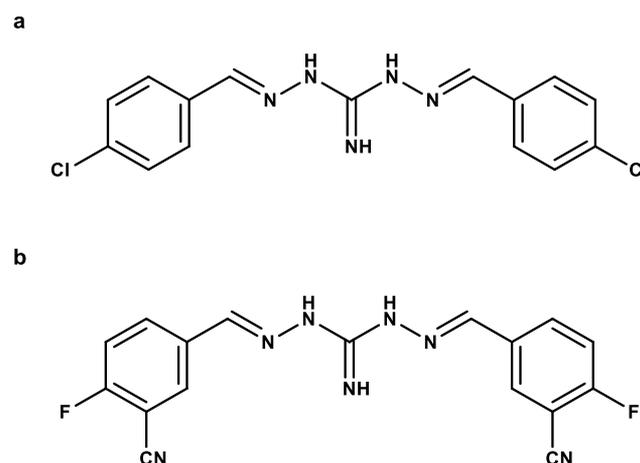
Figure 1. (a) Robenidine, a commercial veterinary drug; (b) **1**, an aminoguanidine with potent antimalarial activity.

Eimeria and *Plasmodium*, the parasite responsible for malaria infections, are both members of the Apicomplexan phylum, distinguished by the presence of the apical complex and frequent presence of the apicoplast organelle.[14]

Robenidine and other aminoguanidines have been evaluated for efficacy against several other protozoan parasites, including *Toxoplasma gondii* (*in vitro* IC$_{50}$ 0.03 μg/mL for robenidine),[15−18] *Leishmania donovani* (*in vitro* IC$_{50}$ 18 μM for the analogue CGP 40215A),[19] *Babesia microti* (murine *in vivo* nonrecrudescence dose 100 mg/kg/day for oral robenidine),[20,21] and the trypanosomes, *T. brucei* and *T. cruzi* (*in vitro* IC$_{50}$ 20 μM for the analogue CGP 40215A).[22−24] It has also been tested against microorganisms such as *Lactobacillus*,[25] *E. coli*,[26] *Acanthamoeba polyphaga*,[27] *Goussia carpelli*,[28] and several other Gram-positive and Gram-negative bacterial pathogens.[29,30]

Recently, work by Trott and McClusky[31−36] has further explored the aminoguanidine chemotype of robenidine, applying a medicinal chemistry approach to repurpose the drug for various bacterial pathogens. Their ongoing research has demonstrated that this chemical scaffold is amenable to synthetic modification and can successfully be refined for activity against pathogens other than *Eimeria*.

Despite significant genetic similarity between *Eimeria* and *Plasmodium*, there has been relatively little research into the efficacy of robenidine against malaria in its 50-year history. Robenidine was evaluated against the murine species *P. berghei* during its initial development in 1970 and found to have moderate activity *in vivo*.[4] An immine analogue of robenidine, CGP 40215A, was tested against the human pathogen *P. falciparum in vitro* with low micromolar IC$_{50}$ value.[37] To date, no medicinal chemistry efforts have focused on refining and optimizing the aminoguanidine scaffold for antimalarial activity.

The current study represents the first attempt to evaluate structure−activity relationships (SAR) of the aminoguanidine chemotype against malaria parasites using *in vitro* assays and an *in vivo* murine model. A library of 38 structurally diverse aminoguanidines was created and compared for *in vitro* antiplasmodial activity as well as mammalian cell cytotoxicity. Compounds with promising selective activity were further evaluated *in vivo* in a murine model of malaria. Multiple aminoguanidines from this library were found to have high potency *in vitro*, which translated to robust efficacy *in vivo*. One compound, 1 (Figure 1b), exhibits single-digit nanomolar IC$_{50}$ values against *P. falciparum* strains with an *in vivo* ED$_{50}$ value below 1 mg/kg/day vs murine malaria.

■ RESULTS AND DISCUSSION

The Aminoguanidine Robenidine Is an Effective Antimalarial *in Vitro*. Robenidine is a symmetrical aminoguanidine drug originally developed for the treatment of *Eimeria*-derived coccidiosis in poultry and livestock.[4] Recent investigations by Trott, McCluskey, and others have demonstrated that the aminoguanidine chemotype can be modified and reapplied to pathogens other than *Eimeria*.[31] Prior to the present study, robenidine had not been tested for antimalarial activity against *P. falciparum* despite the close genetic relationship between *Plasmodium* and *Eimeria*.

To gain an initial assessment of robenidine as a potential antimalarial drug, it was evaluated *in vitro* for the ability to inhibit the growth of *P. falciparum* strain D6, a drug-sensitive strain. Blood stage parasite cultures were incubated in a range

of concentrations of robenidine, and 72 h parasite growth was measured by SYBR green staining relative to untreated controls.[38] In this assessment, robenidine exhibited an average *in vitro* IC$_{50}$ of 324 nM (Figure 2). This value represents an
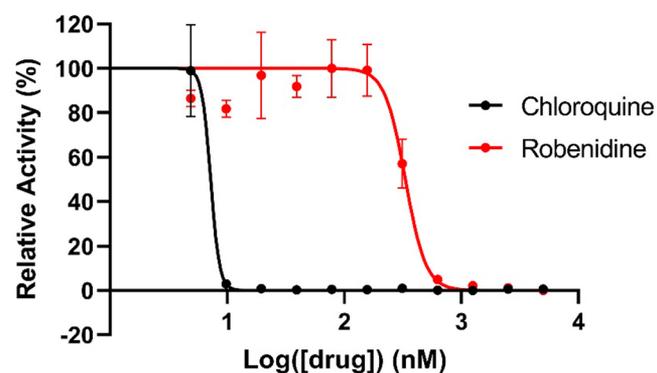
Figure 2. IC$_{50}$ curve of robenidine in D6 compared with chloroquine. Y values represent fluorescence relative to untreated controls.

excellent starting point for further evaluation of structure−activity relationships of robenidine analogues. Notably, the concentration−response curve was characterized by a steep slope at the IC$_{50}$ inflection point, similar to the control drug chloroquine.

Synthesis of Aminoguanidine Robenidine Analogues. Given the successful initial evaluation of robenidine *in vitro*, a series of 38 robenidine analogues was designed and synthesized to determine the potential for improved antimalarial activity within the aminoguanidine chemotype. Robenidine analogues were synthesized in a single step route starting from commercially available substituted benzaldehydes or acetophenones with the synthetic approach utilized in robenidine's initial discovery.[4] Two equivalents of each starting material were refluxed in ethanol with 1,3-diaminoguanidine hydrochloride (Scheme 1). The resulting symmetrical aryl

Scheme 1. A General Synthesis of Aminoguanidine Robenidine Analogues[a]
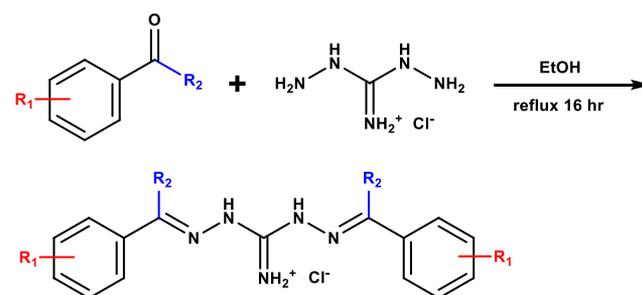
[a]Compounds were prepared by refluxing benzaldehydes or acetophenones with 1,3-diaminoguanidine hydrochloride in ethanol.

aminoguanidine products (formed as HCl salts) were crashed out of solution using diethyl ether, filtered, and washed with diethyl ether. Compounds were purified by recrystallization from ethanol.

As evidenced by the continued commercial success of robenidine as a veterinary drug, this synthetic approach is highly amenable to affordable, large scale synthesis, an important attribute given that any new antimalarial drug

Table 1. Aminoguanidine Halogen Series and Antimalarial Drugs[a]

compound	R_1	R_2	IC$_{50}$ D6 (nM)	IC$_{50}$ Dd2 (nM)	IC$_{50}$ Tm90−C2B (nM)	IC$_{50}$ A6 (nM)	IC$_{50}$ cytotoxicity (μM)	IVTI
2	2-F	H	2866	4593	>5000	1893	>200	>100
3	3-F	H	767	>5000	>5000	426	49	64
4	4-F	H	1016	3792	4904	369	24	24
5	2-Cl	H	334	961	1219	348	>200	>100
6	3-Cl	H	202	567	664	237	35	>100
robenidine	4-Cl	H	324	814	1317	410	30	93
7	2-Br	H	280	715	974	876	94	>100
8	3-Br	H	572	1516	1838	502	62	>100
9	4-Br	H	277	876	899	269	38	>100
atovaquone	N/A	N/A	<1	<1	>5000	>5000	72	>100
chloroquine	N/A	N/A	10	70	297	16	181	>100

[a]See Scheme 1 for the aminoguanidine scaffold and sites of modification. *P. falciparum* IC$_{50}$ values are the average of two to four determinations, each carried out in quadruplicate (a more granular view of this data is provided in the Supporting Information). D6, *P. falciparum* pan-sensitive strain; Dd2, multidrug-resistant *P. falciparum* strain; Tm90−C2B, multidrug-resistant *P. falciparum* clinical isolate that is also resistant to atovaquone; A6, *P. falciparum* in-house derived mutant line resistant to respiratory antagonists.[40] Cytotoxicity assays were carried out with human hepatoma derived HepG2 cells and performed in quadruplicate. IVTI = *in vitro* therapeutic index, defined as cytotoxicity/D6 IC$_{50}$. N/A = not applicable.

Table 2. Aminoguanidine Methyl, Methoxy, Fluoromethyl, Fluoromethoxy, and Nitrile Series[a]

compound	R_1	R_2	IC$_{50}$ D6 (nM)	IC$_{50}$ Dd2 (nM)	IC$_{50}$ Tm90−C2B (nM)	IC$_{50}$ A6 (nM)	IC$_{50}$ cytotoxicity (μM)	IVTI
10	4-CH$_3$	H	905	2427	2529	>5000	43	48
11	4-OCH$_3$	H	263	1602	>5000	289	>200	>100
12	4-CF$_3$	H	614	1536	2742	567	40	65
13	4-CF$_3$	CH$_3$	211	414	451	283	114	>100
14	2-OCF$_3$	H	1080	2328	>5000	391	156	>100
15	3-OCF$_3$	H	670	1571	2988	689	61	91
16	4-OCF$_3$	H	39	83	114	52	11	>100
17	4-OCF$_3$	CH$_3$	140	526	458	182	9	64
18	4-OCHF$_2$	H	71	191	193	87	19	>100
19	4-OCHF$_2$	CH$_3$	99	403	315	171	3	30
20	2-CN	H	1024	2097	1990	969	31	30
21	3-CN	H	7	20	31	24	7	>100
22	4-CN	H	58	166	312	76	>200	>100
23	3-CN	CH$_3$	47	70	104	17	9	>100
1	3-CN, 4-F	H	4	12	16	14	8	>100

[a]See Table 1 legend and Methods.

Table 3. Other Aminoguanidines[a]

compound	R_1	R_2	IC$_{50}$ D6 (nM)	IC$_{50}$ Dd2 (nM)	IC$_{50}$ Tm90−C2B (nM)	IC$_{50}$A6 (nM)	IC$_{50}$ cytotoxicity (μM)	IVTI
24	H	H	705	4298	>5000	581	>200	>100
25	4-OH	H	143	240	286	278	39	>100
26	4-Ph	H	15	87	71	33	>200	>100
27	4-NO$_2$	H	96	198	247	85	>200	>100
28	4-NMe$_2$	H	118	264	213	122	20	>100
29	4-COOH	H	>5000	>5000	>5000	>5000	>200	N/A
30	4-CONH$_2$	H	527	405	727	516	42	80
31	4-SO$_2$NH$_2$	H	>5000	>5000	>5000	>5000	>200	N/A
32	4-propynoxy	H	102	199	376	153	>200	>100
33	4-morpholino	H	131	329	295	116	>200	>100
34	3-tetrazole	H	1645	>5000	>5000	4790	107	65
35	4-tetrazole	H	838	>5000	>5000	>5000	>200	N/A
36	(2,2-difluoro) 2,3-dioxazole	H	250	800	905	235	>200	>100
37	2,4-OMe, 5-Cl	CH$_3$	360	1247	1180	216	3	8
38	mono 2-F[b]	H	1028	2202	>5000	833	>200	>100

[a]See Table 1 legend and Methods. [b]Compound 38 has a 2-fluoro substituent at only one R_1 site. The other R_1 site is unsubstituted (4-H).

must be inexpensive for use in resource poor regions where the disease is endemic. It is noteworthy that the aminoguanidines can be prepared in a single step from commercially available starting materials, and no chromatographic separations are

required for their purification. This is a significant advantage over other antimalarial drug candidates requiring multiple steps and/or complex separations.

Structure−Activity Relationships of Aminoguanidines in Vitro. The *in vitro* activity of each compound in the aminoguanidine library was assessed against asynchronous cultures of *Plasmodium falciparum* parasites replicating within human erythrocytes (Tables 1−3). As the chemical development of robenidine was not discussed in its original publication in 1970[4] and limited structural information is available regarding its mechanism of action,[10] profiling was guided primarily by *in vitro* potency against the *P. falciparum* D6 strain cultured in human erythrocytes as described above.

Robenidine is structured as a diarylaminoguanidine with symmetrical 4-chloro (para relative to the aminoguanidine moiety) substituents on its two phenyl rings. Our SAR studies primarily focused on the potential to improve antimalarial activity by exchanging these 4-chloro substituents for different functional groups at the same position. All compounds are symmetrical and take the form shown in Scheme 1 unless otherwise noted. A halogen series (Table 1) was prepared to examine the effects of both halide type and position on antiplasmodial activity. Within this series, chloro and bromo substituted aminoguanidines were more active than fluoro substituted compounds, with position effects varying among the halides. The ortho-fluoro compound **2** displayed much lower activity than robenidine, and the para-bromo compound **9** showed moderately improved activity.

Other replacement functional groups for robenidine's 4-chloro substituents were varied widely for size, lipophilicity, and electron withdrawing vs donating effects on the adjacent phenyl ring. Compounds with promising activity were also prepared as their ortho and meta isomers to investigate positional effects for these functional groups. Similarly, promising compounds were prepared with additional methyl groups on the benzyl carbons of the aminoguanidine moieties as shown in Scheme 1 (the R_2 position) by starting from the analogous acetophenones rather than benzaldehydes.

The 4-methoxy robenidine derivative **11** was among the earliest compounds to show improved potency over robenidine. To further pursue this activity, the methyl (**10**), trifluoromethyl (**12**), trifluoromethoxy (**16**), and difluoromethoxy (**18**) derivatives were also prepared along with their R_2-methyl analogues (Table 2). Among these compounds **16** was the most potent, quickly becoming the frontrunner with a nearly 10-fold reduction in IC_{50} value (39 nM vs D6) relative to robenidine (324 nM vs D6). Conversion of the R_2 moiety from H to methyl did not have a consistent effect, improving activity for the trifluoromethyl derivative (**12** vs **13**) while reducing activities of the trifluoromethoxy and difluoromethoxy derivatives (**16** vs **17** and **18** vs **19**, respectively). The ortho (**14**) and meta (**15**) variants of **16** were also prepared and demonstrated significantly reduced antimalarial activity in comparison with the para isomer.

The early success of **16** led to an interest in exploring other electron withdrawing groups at the para position. The nitro (**27**) and cyano (**22**) derivatives were significantly more active than robenidine. **22** in particular had activity in the same range as the early hit compound **16**, so the ortho (**20**) and meta (**21**) analogues were prepared to explore the position effects of the nitrile group. This series was expected to show a similar pattern to the trifluoromethoxy compounds, and so it came as quite a surprise when the 3-CN analogue **21** was found to have IC_{50}

value of 7 nM, much lower than any other aminoguanidine evaluated up to that point. The R_2-methyl analogue (**23**) of this compound was prepared and found to be less active than the R_2-H original.

A few general trends were observed for *in vitro* antimalarial activity. In general, substitution at the ortho position dramatically reduced antimalarial activity, possibly by sterically constraining rotation of the aryl rings. The carbonate **29** and tetrazoles **34** and **35** were inactive against all tested *P. falciparum* strains. Electron withdrawing substituents appear to have a positive effect on antimalarial activity, though the biphenyl analogue **26** is unusually potent and the dimethylamino (**28**) and propynoxy (**32**) compounds also exhibit respectable antiparasitic activity.

All aminoguanidines synthesized were also evaluated against three multidrug-resistant *P. falciparum* strains (Dd2, Tm90−C2B, and A6) and for mammalian cell cytotoxicity. Robenidine and the lead compounds **16**, **21**, and **22** were further evaluated *in vivo* to guide optimization, resulting in the design of **1**, the overall series lead. The results of these experiments are listed in Tables 1−3 and discussed below.

Aminoguanidines Retain in Vitro Activity in Drug-Resistant Strains of Malaria. In addition to the drug-sensitive *P. falciparum* D6 strain, the aminoguanidines were assessed against three drug-resistant strains (Tables 1−3). *P. falciparum* Dd2 is a multidrug-resistant strain sensitive to atovaquone but resistant to chloroquine as well as the antifolate combination of pyrimethamine + sulfadoxine. *P. falciparum* Tm90−C2B is a multidrug-resistant clinical isolate including resistance to both atovaquone and chloroquine.[39] *P. falciparum* A6 is derived from D6 and is resistant to respiratory antagonists such as atovaquone and antimycin A but sensitive to chloroquine.[40]

The degree of cross resistance observed for the MDR strains Dd2 and C2B with the tested aminoguanidine series ranged from extensive (e.g., ∼19-fold for **11**), to intermediate (e.g., 6.5-fold for **3**), to modest (2−4-fold for **1** and **23**) relative to the drug sensitive D6 strain of *P. falciparum*. The general lack of significant cross-resistance is consistent with expectations given that robenidine and other aminoguanidines are not clinically prescribed for malaria or even administered directly to humans for any indication (it is possible that trace amounts of robenidine have passed into humans via the consumption of poultry treated for coccidiosis, though these trace amounts are unlikely to drive antimalarial resistance).

Aminoguanidines Have High in Vitro Therapeutic Indices. The aminoguanidines were evaluated for cytotoxicity against human hepatoma derived HepG2 cells (Tables 1-3). In this assay, HepG2 cells were incubated with test compounds for 24 h, followed by a 24-h recovery period and subsequent staining to evaluate for cytotoxic effects with resazurin. The ratio of the resulting HepG2 IC_{50} value to the *P. falciparum* D6 IC_{50} value can be considered an "*in vitro* therapeutic index" or IVTI.

Cytotoxicity in Hep2G did not track proportionally with antimalarial activity in any of the four tested *P. falciparum* strains. Overall, substitution at the 3 (meta) position resulted in increased cytotoxicity relative to the 2 (ortho) and 4 (para) positions, though the cyano substituent was shown to be an exception to this trend. Several of the aminoguanidines, including the active compound **21**, had no measurable effect on Hep2G activity at concentrations as high as 200 μM. Most aminoguanidines in the series had HepG2 IC_{50} values above 10

μM, and nearly all active aminoguanidines had an *in vitro* therapeutic index of over 1000-fold (this value cannot be calculated for those aminoguanidines having no antimalarial activity). It is noteworthy that **1**, with the greatest antiplasmodial activity among compounds in this series, exhibits an IVTI value of 2000, which is indicative of its highly selective antiparasitic action.

Aminoguanidine Activity Is Concentration Dependent but Not Time Dependent. Many drugs have activity dependent on their exposure time in addition to concentration, and antimalarial potency is frequently stage-specific. To determine whether the aminoguanidines acted by a time-dependent mechanism against malaria parasites, the SYBR Green activity assay was adapted to include additional incubation intervals. Two potent aminoguanidines, **16** and **21**, were incubated with *P. falciparum* Dd2 infected erythrocytes for 48, 72, or 96 h (Figure 3). Notably, this
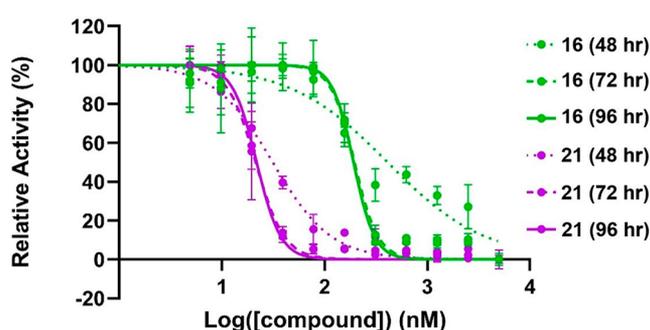
Figure 3. Inhibition of *P. falciparum* Dd2 growth by **16** (green) and **21** (purple) at 48 h (dotted curves), 72 h (dashed curves), and 96 h (solid curves) time points.

assay measures drug-treated parasite growth during the incubation time relative to untreated parasites. A shorter incubation time allows for less parasite growth for all drug conditions, producing data with proportionally greater variability or "noise". Conversely, longer incubation times reduce noise in the resulting data.

IC_{50} values for both **16** and **21** were somewhat higher at 48 h than at longer drug incubation times (Table 4), though this

Table 4. *P. falciparum* Dd2 IC_{50} of 16 and 21 vs Drug Incubation Length[a]

compound	IC_{50} Dd2 (nM)		
	48 h	72 h	96 h
16	410	195	192
21	31	21	22

[a]See Table 1 legend and Methods.

may be the result of noise in the data associated with the short incubation time. This finding may also stem from the use of asynchronous parasite cultures, wherein one life-cycle stage may be more impacted than another (**16** in particular appears to exhibit a biphasic concentration–response curve, indicative of stage specificity). IC_{50} values for both compounds were virtually identical between 72-h and 96-h incubation time points. These results indicate that a 72-h incubation may be required to attain full *in vitro* activity, but that no additional benefit is construed with longer incubation times. Aminoguanidine activity appears to be driven primarily by drug

concentration rather than by lengthening drug incubation time. Additional experiments are planned to explore for possible stage-specific activity of the most active compounds in this series however our results combine to suggest that the molecules are not acting in a manner consistent with a "delayed death" phenotype as shown for other drugs such as doxycycline and azithromycin.[45]

Lead Aminoguanidines Have High *in Vivo* Efficacy in a Mouse Model of Malaria. Aminoguanidines exhibiting high *in vitro* antimalarial potency and robenidine were assessed for *in vivo* efficacy in a murine model of malaria (*Plasmodium yoelii*, Table 5). In this modified 4-day Peters test,[41] mice were

Table 5. *In Vivo* ED_{50} and ED_{90} of Lead Aminoguanidines[a]

compound	*P. yoelii* ED_{50} (mg/kg/day)	*P. yoelii* ED_{90} (mg/kg/day)	*P. yoelii* NRD (mg/kg/day)
robenidine	1.6	4.9	>25
16	1.2	3.7	12.5
22	2.7	7.7	>25
21	7.1	9.4	>25
1	0.25	0.28	>25

[a]*In vivo* activity values were determined from a modified 4-day Peters test. Compounds were administered by oral gavage up to 25 mg/kg/day, near the solubility limit of the PEG delivery vehicle. NRD = nonrecrudescence dose (cure dose).

inoculated with parasites from a donor mouse (day 0), and then dosed orally with drugs on each of the subsequent 4 days (days 1–4). On day 5 of the experiment, the parasitemia for each mouse was determined microscopically by examining methanol-fixed and Giemsa-stained blood smears. The ED_{50} represents the interpolated dose of a compound at which parasitemia was suppressed to one-half that of untreated controls. Similarly, the ED_{90} represents the dose at which parasitemia is suppressed 10-fold. Mice were considered cured of their infection if no parasites were detected in the blood 30 days from the first drug administration, and the nonrecrudescence dose (NRD) represents the lowest dose to achieve a cure.

Robenidine, **16**, **22**, and **21** were evaluated for *in vivo* antimalarial efficacy. That robenidine exhibited respectable in vivo antimalarial activity was somewhat surprising given that it is poorly absorbed and known to accumulate in the gastrointestinal tract.[44] The early hit compound **16** (the 4-OCF_3 analogue, ED_{50} 1.2 mg/kg/day) showed improved *in vivo* activity over robenidine (ED_{50} = 1.6 mg/kg/day), while **22** (the 4-CN analogue, ED_{50} = 2.7 mg/kg/day) did not. Unexpectedly, **21** (the 3-CN analogue, ED_{50} = 7.1 mg/kg/day) was 6-fold less efficacious than **16** *in vivo*, despite being 6-fold more active than **16** *in vitro*. For comparative purposes, consider that the ED_{50} of chloroquine in this system is 1.5 mg/kg/day.[42]

The only aminoguanidine which produced a cure in this model was **16** with NRD of 12.5 mg/kg/day, while other compounds were not curative (including compound **1** described below). It is important to note that failure to produce a cure in this model and by this dosing regimen is not predictive of clinical failure. Indeed, several approved clinical drugs such as chloroquine are not curative in this model at any dose level.

1 is a Highly Potent Antimalarial *in Vitro* and *in Vivo*. The discrepancy between the *in vitro* success (Table 2) and *in*

vivo mediocrity (Table 5) of **21** remained a mystery which we later explored. From previous *in vivo* SAR studies on other scaffolds, we had noted that aryl groups without protective substitutions at the para positions were biologically unstable. While **16** was substituted at the para position, **21** was not, potentially leaving this position vulnerable to hepatic microsomal degradation.

To interrogate this hypothesis, an analogue of **21** was prepared with an additional para-fluoro substituent (**1**, 3-CN, 4-F). Given the low activity of **4** (4-F), this substitution was expected to have little effect on *in vitro* potency while potentially improving upon the *in vivo* activity of **21**. Unexpectedly, **1** had excellent *in vitro* activity, becoming the new series lead in potency. **1** had a single-digit nanomolar IC_{50} value against D6 (4 nM), IC_{50} values in the low double digits for the drug-resistant strains, and an *in vitro* therapeutic index of 2000-fold.

The *in vivo* efficacy of **1** was even more pronounced with an ED_{50} value of 0.25 mg/kg/day, 5-fold lower than its nearest competitor **16**. The single atom difference between **21** (3-CN, 4-H) and **1** (3-CN, 4-F) resulted in a nearly 30-fold improvement in *in vivo* efficacy.

Murine Microsomal Stability of Aminoguanidines Correlates with *in Vivo* Activity. To investigate the relationship between the aminoguanidines' *in vivo* efficacy and their metabolic properties, the murine microsomal stability of a selection of aminoguanidines was evaluated (Table 6).

Table 6. Murine Microsomal Stability of Aminoguanidines[a]

compound	murine microsomal stability, $t^{1/2}$ (min)	predicted Cl_{int} (mL/min/kg)
ketanserin	14.47	377.20
robenidine	158.65	34.40
16	172.16	31.70
21	58.18	93.80
1	186.15	29.32

[a]Data from ChemPartner Co. Ltd., Shanghai, P.R. China. See Methods for full details.

Robenidine, **16**, **21**, **1**, and the control compound ketanserin were incubated with pooled murine liver microsomes and monitored for degradation by LC/MS/MS for 1 h. The concentration vs time plot for each compound was used to determine its biological half-life ($t_{1/2}$) and predicted intrinsic clearance (Cl_{int}).

For all of the aminoguanidines evaluated, murine microsomal stability correlated with *in vivo* efficacy. Robenidine, **16**, and **1** were all biologically stable with half-lives above 150 min (with the same rank order for *in vivo* activity and stability). **21** was metabolically unstable in the presence of murine microsomes, with a half-life of only 58.18 min.

This data supports the notion that **1** is more efficacious *in vivo* than **21** due in part to improved stability. Substituting the 4-position H for F resulted in a threefold increase in metabolic stability. Presumably the prolonged presence of **1** in the bloodstream contributes to its excellent performance *in vivo*.

CONCLUSIONS

1 is a robenidine derivative with excellent *in vitro* potency, virtually no cross-resistance in multidrug-resistant strains, and a high *in vitro* therapeutic index. In a murine model of malaria, **1** displayed robust *in vivo* antimalarial activity propelled by a combination of high intrinsic potency and biological stability. Although speculative at this time, it is also possible that the nitrogen atoms in the aminoguanidine bridge of **1** exhibit diminished basicity (i.e., ionizability) due to the presence of two strongly electron withdrawing groups (F and CN) at the para and meta positions of the flanking aromatic rings, which may in turn enhance oral bioavailability.

Further exploration of the aminoguanidine scaffold is certainly warranted, as is developing more knowledge of its antimalarial properties, including the mode of action. Assessing the *in vivo* activity of these compounds in humanized mice may refine predictions of clinical success. Assessing the compounds against synchronous parasites will elucidate potential stage-specific potency effects. Beyond the blood stage, evaluating the aminoguanidines against other stages of the malaria life cycle such as the liver stage will provide valuable information useful for their potential development for use in humans. Systematically exploring the mechanism of action, including chemical biology techniques and resistance studies, can further guide SAR for this chemotype, and we are currently engaged in this work. Potential synergy with other antimalarial compounds such as artemisinin, atovaquone, and ELQ-300 will also be assessed.

As we look for new entries in the antimalarial pipeline, it may be useful to reexamine drugs and chemotypes effective in related parasites and pathogens. This appears to be the case with robenidine, a drug discovered in 1970 but which has not been methodically explored in malaria using a medicinal chemistry approach until this point. Though robenidine itself has reasonably good antimalarial activity *in vitro* and *in vivo*, it did not take long to improve upon this activity in both settings. In this study, a second look at an old drug efficiently produced a new and promising chemical lead.

METHODS

General Chemistry: Materials and Instruments. All solvents, starting materials, and reagents were acquired from commercial sources (Sigma-Aldrich and Combi-Blocks). Robenidine was obtained from Santa Cruz Biotechnology (Santa Cruz, California). All materials were used without further purification. ^{1}H and ^{13}C NMR spectra were taken on a Bruker 400 MHz instrument, and chemical shifts are reported relative to TMS (0.0 ppm). Fluorescence measurements were recorded using a Molecular Devices Spectramax iD3 equipped with Softmax Pro 7 software. Final compounds were measured to be >95% pure by high performance liquid chromatography (HPLC) using an Agilent Technologies 1260 Infinity II system (unless otherwise noted). High-resolution mass spectrometry (HRMS) using electrospray ionization was performed by the Portland State University BioAnalytical Mass Spectrometry Facility. Melting points were measured using a Stanford Research Systems OptiMelt Automated Melting Point System (model MPA100).

1: 2,2′-Bis[(3-cyano-4-fluorophenyl)methylene]-carbonimidic Dihydrazide Hydrochloride. A solution of 3-cyano-4-fluorobenzaldehyde (1.31 g, 8.8 mmol, 2.2 equiv) and 1,3-diaminoguanidine hydrochloride (500 mg, 4 mmol, 1 equiv) in ethanol (5 mL) was refluxed for 16 h. Diethyl ether (10 mL) was added, and the product carbonimidic dihydrazide crashed out of solution as a white solid. The product was filtered, washed with diethyl ether, and recrystallized from methanol as a hydrochloride salt. Yield: 1.54 g, 99%. ^{1}H NMR (400 MHz, DMSO-d6) δ 12.56 (s, 2H), 8.76 (s, 2H), 8.64 (d,

J = 5.63 Hz, 2H), 8.46 (s, 2H), 8.33−8.32 (m, 2H), 7.68 (t, J = 8.92, 2H). ^{13}C NMR (400 MHz, DMSO-d6) δ 164.98, 162.40, 153.57, 146.25, 136.94, 135.95, 133.22, 131.61, 131.57, 117.73, 117.53, 114.22, 101.56, 101.40. HRMS found 352.1111, M+H. MP = 317−319 °C.

2: 2,2′-Bis[(2-fluorophenyl)methylene]carbonimidic Dihydrazide Hydrochloride. A solution of 2-fluorobenzaldehyde (1.1 g, 8.8 mmol, 2.2 equiv) and 1,3-diaminoguanidine hydrochloride (500 mg, 4 mmol, 1 equiv) in ethanol (5 mL) was refluxed for 16 h. Diethyl ether (10 mL) was added, and the product carbonimidic dihydrazide crashed out of solution as a white solid. The product was filtered, washed with diethyl ether, and recrystallized from methanol as a hydrochloride salt. Yield: 1.34 g, 99%. ^{1}H NMR (400 MHz, DMSO-d6) δ 12.54 (s, 2H), 8.72 (s, 2H), 8.64 (s, 2H), 8.36 (dt, J = 7.9, 1.72, 2H), 7.59−7.53 (m, 2H), 7.34 (t, J = 7.9 Hz, 4H). ^{13}C NMR (400 MHz, DMSO-d6) δ 162.21, 159.71, 152.74, 141.62, 132.93, 127.31, 124.75, 120.82, 116.07, 115.86. HRMS found 302.1206, M+H. MP = 293−295 °C.

3: 2,2′-Bis[(3-fluorophenyl)methylene]carbonimidic Dihydrazide Hydrochloride. A solution of 3-fluorobenzaldehyde (1.09 g, 8.8 mmol, 2.2 equiv) and 1,3-diaminoguanidine hydrochloride (500 mg, 4 mmol, 1 equiv) in ethanol (5 mL) was refluxed for 16 h. Diethyl ether (10 mL) was added, and the product carbonimidic dihydrazide crashed out of solution as a white solid. The product was filtered, washed with diethyl ether, and recrystallized from methanol as a hydrochloride salt. Yield: 1.31 g, 97%. ^{1}H NMR (400 MHz, DMSO-d6) δ 12.56 (s, 2H), 8.69 (s, 2H), 8.48 (s, 2H), 7.98 (d, J = 10.3 Hz, 2H), 7.70(d, 7.6 Hz, 2H), 7.53 (q, J = 7.3, 2H), 7.33 (t, J = 8.3, 2H). ^{13}C NMR (400 MHz, DMSO-d6) δ 164.14, 161.72, 153.47, 148.02, 136.37, 136.28, 131.34, 131,26, 125.37, 118.15, 117.94, 113.89, 113.66. HRMS found 302.1206, M+H. MP = 281−283 °C.

4: 2,2′-Bis[(4-fluorophenyl)methylene]carbonimidic Dihydrazide Hydrochloride. A solution of 4-fluorobenzaldehyde (1.09 g, 8.8 mmol, 2.2 equiv) and 1,3-diaminoguanidine hydrochloride (500 mg, 4 mmol, 1 equiv) in ethanol (5 mL) was refluxed for 16 h. Diethyl ether (10 mL) was added, and the product carbonimidic dihydrazide crashed out of solution as a white solid. The product was filtered, washed with diethyl ether, and recrystallized from methanol as a hydrochloride salt. Yield: 1.33 g, 99%. ^{1}H NMR (400 MHz, DMSO-d6) δ 12.32 (s, 2H), 8.56 (s, 2H), 8.44 (s, 2H), 8.03 (t, J = 6.5 Hz, 4H), 7.34 (t, J = 8.2, 4H). ^{13}C NMR (400 MHz, DMSO-d6) δ 165.24, 162.77, 153.41, 148.14, 130.71, 130.62, 130.48, 130.45, 116.40, 116.19. HRMS found 302.1205, M+H. MP = 295−297 °C.

5: 2,2′-Bis[(2-chlorophenyl)methylene]carbonimidic Dihydrazide Hydrochloride. A solution of 2-chlorobenzaldehyde (1.23 g, 8.8 mmol, 2.2 equiv) and 1,3-diaminoguanidine hydrochloride (500 g, 4 mmol, 1 equiv) in ethanol (5 mL) was refluxed for 16 h. Diethyl ether (10 mL) was added, and the product carbonimidic dihydrazide crashed out of solution as a white solid. The product was filtered, washed with diethyl ether, and recrystallized from methanol as a hydrochloride salt. Yield: 1.42 g, 96%. ^{1}H NMR (400 MHz, DMSO-d6) δ 12.62 (s, 2H), 8.88 (s, 2H), 8.69 (s, 2H), 8.43 (d, J = 7.15 Hz, 2H), 7.58−7.46 (m, 6H). ^{13}C NMR (400 MHz, DMSO-d6) δ 153.19, 145.53, 134.06, 132.77, 131.03, 130.39, 128.51, 127.95. HRMS found 334.0620, M+H. MP = 295−297 °C.

6: 2,2′-Bis[(3-chlorophenyl)methylene]carbonimidic Dihydrazide Hydrochloride. A solution of 3-chlorobenzaldehyde

(1.23 g, 8.8 mmol, 2.2 equiv) and 1,3-diaminoguanidine hydrochloride (500 g, 4 mmol, 1 equiv) in ethanol (5 mL) was refluxed for 16 h. Diethyl ether (10 mL) was added, and the product carbonimidic dihydrazide crashed out of solution as a white solid. The product was filtered, washed with diethyl ether, and recrystallized from methanol as a hydrochloride salt. Yield: 1.48 g, 100%. ^{1}H NMR (400 MHz, DMSO-d6) δ 12.39 (s, 2H), 8.69 (s, 2H), 8.43 (s, 2H), 8.17 (s, 2H), 7.83 (d, J = 6.49 Hz, 2H), 7.59−7.50 (m, 2H). ^{13}C NMR (400 MHz, DMSO-d6) δ 153.40, 147.93, 135.91, 134.27, 131.11. 130.92, 127.71, 127.06. HRMS found 334.0619, M+H. MP = 268−270 °C.

7: 2,2′-Bis[(2-bromophenyl)methylene]carbonimidic Dihydrazide Hydrochloride. A solution of 2-bromobenzaldehyde (1.23 g, 8.8 mmol, 2.2 equiv) and 1,3-diaminoguanidine hydrochloride (500 g, 4 mmol, 1 equiv) in ethanol (5 mL) was refluxed for 16 h. Diethyl ether (10 mL) was added, and the product carbonimidic dihydrazide crashed out of solution as a white solid. The product was filtered, washed with diethyl ether, and recrystallized from methanol as a hydrochloride salt. Yield: 1.83 g, 100%. ^{1}H NMR (400 MHz, DMSO-d6) δ 12.52 (s, 2H), 8.80 (s, 2H), 8.66 (s, 2H), 8.39 (d, J = 7.64 Hz, 2H), 7.73 (d, J = 7.94, 2H), 7.51 (t, J = 7.43 Hz, 2H), 7.43 (t, J = 7.56, 2H). ^{13}C NMR (400 MHz, DMSO-d6) δ 153.21, 133.66, 133.00, 132.53, 128.87, 128.44, 124.44. HRMS found 423.9586, M+H. MP = 279−281 °C.

8: 2,2′-Bis[(3-bromophenyl)methylene]carbonimidic Dihydrazide Hydrochloride. A solution of 3-bromobenzaldehyde (1.63 g, 8.8 mmol, 2.2 equiv) and 1,3-diaminoguanidine hydrochloride (500 g, 4 mmol, 1 equiv) in ethanol (5 mL) was refluxed for 16 h. Diethyl ether (10 mL) was added, and the product carbonimidic dihydrazide crashed out of solution as a white solid. The product was filtered, washed with diethyl ether, and recrystallized from methanol as a hydrochloride salt. Yield: 1.80 g, 98%. ^{1}H NMR (400 MHz, DMSO-d6) δ 12.28 (s, 2H), 8.66 (s, 2H), 8.39 (s, 2H), 8.29 (t, J = 3.08 Hz, 2H), 7.87 (d, J = 7.80, 2H), 7.68 (dd, J = 8.00, 2.79, 2H), 7.45 (t, J = 7.86, 2H). ^{13}C NMR (400 MHz, DMSO-d6) δ 136.13, 133.80, 131.34, 129.95, 128.05, 122.83. HRMS found 423.9589, M+H. MP = 258−260 °C.

9: 2,2′-Bis[(4-bromophenyl)methylene]carbonimidic Dihydrazide Hydrochloride. A solution of 4-bromobenzaldehyde (1.63 g, 8.8 mmol, 2.2 equiv) and 1,3-diaminoguanidine hydrochloride (500 g, 4 mmol, 1 equiv) in ethanol (5 mL) was refluxed for 16 h. Diethyl ether (10 mL) was added, and the product carbonimidic dihydrazide crashed out of solution as a white solid. The product was filtered, washed with diethyl ether, and recrystallized from methanol as a hydrochloride salt. Yield: 1.73 g, 94%. ^{1}H NMR (400 MHz, DMSO-d6) δ 12.32 (s, 2H), 8.61 (s, 2H), 8.41 (s, 2H), 7.91 (d, J = 8.24 Hz, 4H), 7.70 (d, J = 8.21, 4H). ^{13}C NMR (400 MHz, DMSO-d6) δ 153.32, 148.29, 133.05, 132.23, 130.23, 124.70. HRMS found 423.9587, M+H. MP = 295−297 °C.

10: 2,2′-Bis[(4-methylphenyl)methylene]carbonimidic Dihydrazide Hydrochloride. A solution of 4-methylbenzaldehyde (1.06 g, 8.8 mmol, 2.2 equiv) and 1,3-diaminoguanidine hydrochloride (500 mg, 4 mmol, 1 equiv) in ethanol (5 mL) was refluxed for 16 h. Diethyl ether (10 mL) was added, and the product carbonimidic dihydrazide crashed out of solution as a white solid. The product was filtered, washed with diethyl ether, and recrystallized from methanol as a hydrochloride salt. Yield: 1.11 g, 84%. ^{1}H NMR (400 MHz, DMSO-d6) δ 12.18 (s, 2H), 8.46 (s, 2H), 8.39 (s, 2H), 7.83 (d, J = 7.8 Hz, 4H),

7.30 (d, J = 7.8 Hz, 4H), 2.37 (s, 6H). ^{13}C NMR (400 MHz, DMSO-d6) δ 152.08, 148.22, 140.13, 130.00, 128.74, 127.24, 20.51. HRMS found 294.1708, M+H. MP = 246−248 °C.

11: 2,2′-Bis[(4-methoxyphenyl)methylene]carbonimidic Dihydrazide Hydrochloride. A solution of 4-methoxybenzaldehyde (1.2 g, 8.8 mmol, 2.2 equiv) and 1,3-diaminoguanidine hydrochloride (500 mg, 4 mmol, 1 equiv) in ethanol (5 mL) was refluxed for 16 h. Diethyl ether (10 mL) was added, and the product carbonimidic dihydrazide crashed out of solution as a white solid. The product was filtered, washed with diethyl ether, and recrystallized from methanol as a hydrochloride salt. Yield: 1.37 g, 94%. ^{1}H NMR (400 MHz, DMSO-d6) δ 12.00 (s, 2H), 8.37 (s, 2H), 8.35 (s, 2H), 7.88 (d, J = 9.1 Hz, 4H), 7.04 (d, J = 9.1 Hz, 4H), 3.83 (s, 6H). ^{13}C NMR (400 MHz, DMSO-d6) δ 181.34, 152.55, 129.56, 125.90, 114.22, 55.38. HRMS found 326.1608, M+H. MP = 218−220 °C.

12: 2,2′-Bis[(4-trifluoromethylphenyl)methylene]-carbonimidic Dihydrazide Hydrochloride. A solution of trifluoromethylbenzaldehyde (1.53 g, 8.8 mmol, 2.2 equiv) and 1,3-diaminoguanidine hydrochloride (500 mg, 4 mmol, 1 equiv) in ethanol (5 mL) was refluxed for 16 h. Diethyl ether (10 mL) was added, and the product carbonimidic dihydrazide crashed out of solution as a white solid. The product was filtered, washed with diethyl ether, and recrystallized from methanol as a hydrochloride salt. Yield: 1.75 g, 100%. ^{1}H NMR (400 MHz, DMSO-d6) δ 12.55 (s, 2H), 8.78 (s, 2H), 8.55 (s, 2H), 8.19 (d, J = 7.8 Hz, 4H), 7.85 (d, J = 8.1, 4H). ^{13}C NMR (400 MHz, DMSO-d6) δ 153.12, 147.44, 137.21, 130.44, 130.12, 128.51, 125.53, 125.39, 122.68. HRMS found 402.1140, M+H. MP = 273−275 °C.

13: 2,2′-Bis[(4-trifluoromethylphenyl)ethylidene]-carbonimidic Dihydrazide Hydrochloride. A solution of 4-trifluoromethylacetophenone (1.65 g, 8.8 mmol, 2.2 equiv) and 1,3-diaminoguanidine hydrochloride (500 mg, 4 mmol, 1 equiv) in ethanol (5 mL) was refluxed for 16 h. Diethyl ether (10 mL) was added, and the product carbonimidic dihydrazide crashed out of solution as a white solid. The product was filtered, washed with diethyl ether, and recrystallized from methanol as a hydrochloride salt. Yield: 1.78 g, 96%. ^{1}H NMR (400 MHz, DMSO-d6) δ 12.04 (s, 2H), 8.89 (s, 2H), 8.28 (d, J = 7.6 Hz, 4H), 7.81 (d, J = 8.4, 4H), 2.5 (s, 6H). ^{13}C NMR (400 MHz, DMSO-d6) δ 154.75, 141.00, 128.26, 125.63, 123.28. HRMS found 430.1455, M+H. MP = 334−336 °C.

14: 2,2′-Bis[(2-trifluoromethoxyphenyl)methylene]-carbonimidic Dihydrazide Hydrochloride. A solution of 2-trifluoromethoxybenzaldehyde (1.67 g, 8.8 mmol, 2.2 equiv) and 1,3-diaminoguanidine hydrochloride (500 mg, 4 mmol, 1 equiv) in ethanol (5 mL) was refluxed for 16 h. Diethyl ether (10 mL) was added, and the product carbonimidic dihydrazide crashed out of solution as a white solid. The product was filtered, washed with diethyl ether, and recrystallized from methanol as a hydrochloride salt. Yield: 1.87 g, 100%. ^{1}H NMR (400 MHz, DMSO-d6) δ 12.54 (s, 2H), 8.75 (s, 2H), 8.71 (s, 2H), 8.48 (d, J = 7.8 Hz, 2H), 7.67−7.50 (m, 6H). ^{13}C NMR (400 MHz, DMSO-d6) δ 152.21, 146.36, 131.98, 127.28, 127.14, 125.67, 121.03, 120.74, 118.19. HRMS found 434.1039, M+H. MP = 194−196 °C.

15: 2,2′-Bis[(3-trifluoromethoxyphenyl)methylene]-carbonimidic Dihydrazide Hydrochloride. A solution of 3-trifluoromethoxybenzaldehyde (835 mg, 4.4 mmol, 2.2 equiv) and 1,3-diaminoguanidine hydrochloride (250 mg, 2 mmol, 1 equiv) in ethanol (5 mL) was refluxed for 16 h. Diethyl ether (10 mL) was added, and the product carbonimidic dihydrazide

crashed out of solution as a white solid. The product was filtered, washed with diethyl ether, and recrystallized from methanol as a hydrochloride salt. Yield: 660 mg, 70%. ^{1}H NMR (400 MHz, DMSO-d6) δ 12.43, (s, 2H), 8.71 (s, 2H), 8.47 (s, 2H), 8.09 (s, 2H), 7.93 (d, J = 7.6 Hz, 2H), 7.63 (t, J = 7.9, 2H), 7.49 (d, J = 8.3 Hz, 2H). ^{13}C NMR (400 MHz, DMSO-d6) δ 153.48, 149.29, 147.93, 136.22, 131.31, 128.06, 123.45, 121.84, 120.13. HRMS found 434.1039, M+H. MP = 241−243 °C.

16: 2,2′-Bis[(4-trifluoromethoxyphenyl)methylene]-carbonimidic Dihydrazide Hydrochloride. A solution of 4-trifluoromethoxybenzaldehyde (1.67 g, 8.8 mmol, 2.2 equiv) and 1,3-diaminoguanidine hydrochloride (500 mg, 4 mmol, 1 equiv) in ethanol (5 mL) was refluxed for 16 h. Diethyl ether (10 mL) was added, and the product carbonimidic dihydrazide crashed out of solution as a white solid. The product was filtered, washed with diethyl ether, and recrystallized from methanol as a hydrochloride salt. Yield: 1.03 g, 55%. ^{1}H NMR (400 MHz, DMSO-d6) δ 12.24 (s, 2H), 8.61 (s, 2H), 8.45 (s, 2H), 8.10 (d, J = 8.9 Hz, 4H), 7.50 (d, J = 8.1 Hz, 4H). ^{13}C NMR (400 MHz, DMSO-d6) δ 150.26, 133.07, 130.34, 121.70. HRMS found 434.1041, M+H. MP = 278−280 °C.

17: 2,2′-Bis[(4-trifluoromethoxyphenyl)ethylidene]-carbonimidic Dihydrazide Hydrochloride. A solution of 4-trifluoromethoxyacetophenone (1.79 g, 8.8 mmol, 2.2 equiv) and 1,3-diaminoguanidine hydrochloride (500 mg, 4 mmol, 1 equiv) in ethanol (5 mL) was refluxed for 16 h. Diethyl ether (10 mL) was added, and the product carbonimidic dihydrazide crashed out of solution as a white solid. The product was filtered, washed with diethyl ether, and recrystallized from methanol as a hydrochloride salt. Yield: 1.88 g, 95%. ^{1}H NMR (400 MHz, DMSO-d6) δ 11.91 (s, 2H), 8.78 (s, 2H), 8.19 (d, J = 8.8 Hz, 4H), 7.44 (d, J = 8.1, 4H), 2.46 (s, 6H). ^{13}C NMR (400 MHz, DMSO-d6) δ 154.66, 149.85, 136.38, 129.60, 121.79, 121.17, 119.24. HRMS found 462.1352, M+H. MP = 323−325 °C.

18: 2,2′-Bis[(4-difluoromethoxyphenyl)methylene]-carbonimidic Dihydrazide Hydrochloride. A solution of 4-difluoromethoxybenzaldehyde (757 mg, 4.4 mmol, 2.2 equiv) and 1,3-diaminoguanidine hydrochloride (250 g, 2 mmol, 1 equiv) in ethanol (5 mL) was refluxed for 16 h. Diethyl ether (10 mL) was added, and the product carbonimidic dihydrazide crashed out of solution as a white solid. The product was filtered, washed with diethyl ether, and recrystallized from methanol as a hydrochloride salt. Yield: 0.63 g, 73%. ^{1}H NMR (400 MHz, DMSO-d6) δ 12.28 (s, 2H), 8.56 (s, 2H), 8.43 (s, 2H), 8.02 (d, J = 8.6, 4H), 7.38 (t, J = 74, 2H), 7.29 (d, J = 8.6 Hz, 4H). ^{13}C NMR (400 MHz, DMSO-d6) δ 153.17, 130.67, 130.22, 119.01, 116.58. HRMS found 398.1231, M+H. MP = 245−247 °C.

19: 2,2′-Bis[(4-difluoromethoxyphenyl)ethylidene]-carbonimidic Dihydrazide Hydrochloride. A solution of 4-difluoromethoxyacetophenone (818 mg, 4.4 mmol, 2.2 equiv) and 1,3-diaminoguanidine hydrochloride (250 mg, 2 mmol, 1 equiv) in ethanol (5 mL) was refluxed for 16 h. Diethyl ether (10 mL) was added, and the product carbonimidic dihydrazide crashed out of solution as a white solid. The product was filtered, washed with diethyl ether, and recrystallized from methanol as a hydrochloride salt. Yield: 990 mg, 100%. ^{1}H NMR (400 MHz, DMSO-d6) δ 11.78 (s, 2H), 8.72 (s, 2H), 8.13 (d, J = 8.8 Hz, 4H), 7.36 (t, J = 73.56, 2H), 7.24 (d, J = 8.6, 2H). ^{13}C NMR (400 MHz, DMSO-d6) δ 154.53, 152.90,

152.66, 133.97, 129.39, 119.19, 118.54, 116.62, 114.06, 15.33. HRMS found 426.1538, M+H. MP = 273−275 °C.

20: 2,2′-Bis[(2-cyanophenyl)methylene]carbonimidic Dihydrazide Hydrochloride. A solution of 2-cyanobenzaldehyde (1.15 g, 8.8 mmol, 2.2 equiv) and 1,3-diaminoguanidine hydrochloride (500 mg, 4 mmol, 1 equiv) in ethanol (5 mL) was refluxed for 16 h. Diethyl ether (10 mL) was added, and the product carbonimidic dihydrazide crashed out of solution as a white solid. The product was filtered, washed with diethyl ether, and recrystallized from methanol as a hydrochloride salt. Yield: 1.23 g, 88%. ^{1}H NMR (400 MHz, DMSO-d6) δ 12.88 (s, 2H), 8.8 (s, 2H), 8.74 (s, 2H), 8.52 (d, J = 7.8 Hz, 2H), 7.96 (d, J = 7.8 Hz, 2H), 7.84 (t, J = 7.7 Hz, 2H), 7.68 (t, J = 7.7, 2H), 7.34 (t, J = 50.59, 2H). ^{13}C NMR (400 MHz, DMSO-d6) δ 145.01. 136.32, 133.91, 133.88, 131.56, 127.55, 117.67, 111.51, 56.48, 19.03. HRMS found 316.1301, M+H. MP = 213-215 °C.

21: 2,2′-Bis[(3-cyanophenyl)methylene]carbonimidic Dihydrazide Hydrochloride. A solution of 3-cyanobenzaldehyde (1.15 g, 8.8 mmol, 2.2 equiv) and 1,3-diaminoguanidine hydrochloride (500 mg, 4 mmol, 1 equiv) in ethanol (5 mL) was refluxed for 16 h. Diethyl ether (10 mL) was added, and the product carbonimidic dihydrazide crashed out of solution as a white solid. The product was filtered, washed with diethyl ether, and recrystallized from methanol as a hydrochloride salt. Yield: 1.40 g, 100%. ^{1}H NMR (400 MHz, DMSO-d6) δ 12.44 (s, 2H), 8.74 (s, 2H), 8.56 (s, 2H), 8.46 (s, 2H), 8.22 (d, J = 8.1 Hz, 2H), 7.95 (d, J = 7.8 Hz, 2H), 7.71 (t, J = 7.6 Hz, 2H). ^{13}C NMR (400 MHz, DMSO-d6) δ 153.60, 147.27, 135.10, 134.21, 133.25, 131.24, 130.50, 118.95, 112.49. HRMS found 316.1300, M+H. MP = 278−280 °C.

22: 2,2′-Bis[(4-cyanophenyl)methylene]carbonimidic Dihydrazide Hydrochloride. A solution of 4-cyanobenzaldehyde (1.15 g, 8.8 mmol, 2.2 equiv) and 1,3-diaminoguanidine hydrochloride (500 mg, 4 mmol, 1 equiv) in ethanol (5 mL) was refluxed for 16 h. Diethyl ether (10 mL) was added, and the product carbonimidic dihydrazide crashed out of solution as a white solid. The product was filtered, washed with diethyl ether, and recrystallized from methanol as a hydrochloride salt. Yield: 1.40 g, 100%. ^{1}H NMR (400 MHz, DMSO-d6) δ 12.69 (s, 2H), 8.79 (s, 2H), 8.52 (s, 2H), 8.17 (d, J = 8.2 Hz, 4H), 7.97 (d, J = 8.3 Hz, 4H). ^{13}C NMR (400 MHz, DMSO-d6) δ 153.64, 147.70, 138.14, 133.11, 128.92, 119.09, 113.05. HRMS found 316.1303, M+H. MP = 303−305 °C.

23: 2,2′-Bis[(3-cyanophenyl)ethylidene]carbonimidic Dihydrazide Hydrochloride. A solution of 3-cyanoacetophenone (1.28 g, 8.8 mmol, 2.2 equiv) and 1,3-diaminoguanidine hydrochloride (500 mg, 4 mmol, 1 equiv) in ethanol (5 mL) was refluxed for 16 h. Diethyl ether (10 mL) was added, and the product carbonimidic dihydrazide crashed out of solution as a white solid. The product was filtered, washed with diethyl ether, and recrystallized from methanol as a hydrochloride salt. Yield: 1.40 g, 92%. ^{1}H NMR (400 MHz, DMSO-d6) δ 11.89 (s, 2H), 8.90 (s, 2H), 8.63 (t, J = 1.44 Hz, 2H), 8.33 (dt, J = 7.75, 1.39, 2H), 7.92 (dt, J = 7.76, 2.44, 2H), 7.67 (t, J = 7.88, 2H), 2.48 (s, 6H). ^{13}C NMR (400 MHz, DMSO-d6) δ 154.70, 152.16, 138.21, 133.59, 132.06, 131.04, 130.09, 119.21, 112.16, 15.29. HRMS found 344.1612, M+H. MP = 304−306 °C.

24: 2,2′-Bis(phenylmethylene)carbonimidic Dihydrazide Hydrochloride. A solution of benzaldehyde (933 mg, 8.8 mmol, 2.2 equiv) and 1,3-diaminoguanidine hydrochloride (500 mg, 4 mmol, 1 equiv) in ethanol (5 mL) was refluxed for 16 h. Diethyl ether (10 mL) was added, and the product

carbonimidic dihydrazide crashed out of solution as a white solid. The product was filtered, washed with diethyl ether, and recrystallized from methanol as a hydrochloride salt. Yield: 1.19 g, 99%. ^{1}H NMR (400 MHz, DMSO-d6) δ 12.23 (s, 2H), 8.53 (s, 2H), 8.44 (s, 2H), 7.96−7.94 (m, 4H), 7.50−7.49 (m, 6H). ^{13}C NMR (400 MHz, DMSO-d6) δ 133.27, 130.77, 128.73, 127.86. HRMS found 266.1398, M+H. MP = 245−247 °C.

25: 2,2′-Bis[(4-phenol)methylene]carbonimidic Dihydrazide Hydrochloride. A solution of 4-hydroxybenzaldehyde (1.1 g, 8.8 mmol, 2.2 equiv) and 1,3-diaminoguanidine hydrochloride (500 mg, 4 mmol, 1 equiv) in ethanol (5 mL) was refluxed for 16 h. Diethyl ether (10 mL) was added, and the product carbonimidic dihydrazide crashed out of solution as a white solid. The product was filtered, washed with diethyl ether, and recrystallized from methanol as a hydrochloride salt. Yield: 1.33 g, 100%. ^{1}H NMR (400 MHz, DMSO-d6) δ 11.95 (s, 2H), 10.15 (s, 2H), 8.28 (s, 2H), 7.75 (d, J = 8.1 Hz, 4H), 6.87 (d, J = 8.3, 4H). ^{13}C NMR (400 MHz, DMSO-d6) δ 160.56, 130.15, 124.76, 116.10. HRMS found 298.1296, M+H. MP = 185−187 °C.

26: 2,2′-Bis[(4-diphenyl)methylene]carbonimidic Dihydrazide Hydrochloride. A solution of 4-phenylbenzaldehyde (1.60 g, 8.8 mmol, 2.2 equiv) and 1,3-diaminoguanidine hydrochloride (500 mg, 4 mmol, 1 equiv) in ethanol (5 mL) was refluxed for 16 h. Diethyl ether (10 mL) was added, and the product carbonimidic dihydrazide crashed out of solution as a white solid. The product was filtered, washed with diethyl ether, and recrystallized from methanol as a hydrochloride salt. Yield: 1.81 g, 100%. ^{1}H NMR (400 MHz, DMSO-d6) δ 12.32 (s, 2H), 8.59 (s, 2H), 8.49 (s, 2H), 8.05 (d, J = 7.3 Hz, 2H), 7.81 (d, J = 7.3 Hz, 2H), 7.77 (d, J = 7.6 Hz, 2H), 7.51 (t, J = 7.3 Hz, 2H), 7.42 (t, J = 7.6 Hz, 1H). ^{13}C NMR (400 MHz, DMSO-d6) δ 153.28, 142.71, 139.72, 132.89, 129.52, 128.99, 128.51, 127.42, 127.28. HRMS found 418.2022, M+H. MP = 286−288 °C.

27: 2,2′-Bis[(4-nitrophenyl)methylene]carbonimidic Dihydrazide Hydrochloride. A solution of 4-nitrobenzaldehyde (1.33 g, 8.8 mmol, 2.2 equiv) and 1,3-diaminoguanidine hydrochloride (500 mg, 4 mmol, 1 equiv) in ethanol (5 mL) was refluxed for 16 h. Diethyl ether (10 mL) was added, and the product carbonimidic dihydrazide crashed out of solution as a white solid. The product was filtered, washed with diethyl ether, and recrystallized from methanol as a hydrochloride salt. Yield: 1.56 g, 100%. ^{1}H NMR (400 MHz, DMSO-d6) δ 12.68 (s, 2H), 8.82 (s, 2H), 8.57 (s, 2H), 8.33 (d, J = 8.3 Hz, 4H), 8.24 (d, J = 8.3 Hz, 4H). ^{13}C NMR (400 MHz, DMSO-d6) δ 153.79, 148.78, 147.27, 139.98, 129.35, 124.35. HRMS found 356.1098, M+H. MP = 285−287 °C.

28: 2,2′-Bis[(4-dimethylaminophenyl)methylene]carbonimidic Dihydrazide Hydrochloride. A solution of *N,N*-dimethylaminobenzaldehyde (1.31 g, 8.8 mmol, 2.2 equiv) and 1,3-diaminoguanidine hydrochloride (500 mg, 4 mmol, 1 equiv) in ethanol (5 mL) was refluxed for 16 h. Diethyl ether (10 mL) was added, and the product carbonimidic dihydrazide crashed out of solution as a white solid. The product was filtered, washed with diethyl ether, and recrystallized from methanol as a hydrochloride salt. Yield: 90 mg, 6%. This compound was chemically unstable in chromatography solvents but was greater than 80% pure when used in *in vitro* assays. ^{1}H NMR (400 MHz, DMSO-d6) δ 11.74 (s, 2H), 8.22 (s, 2H), 8.14 (s, 2H), 7.71 (d, J = 7.8 Hz, 4H), 6.75 (d, J = 7.8 Hz, 4H), 3.00 (s, 6H). ^{13}C NMR (400

MHz, DMSO-d6) δ 152.50, 152.35, 129.69, 129.41, 121.22, 120.96, 112.01. HRMS found 352.2237, M+H. MP = 132−134 °C.

29: 2,2′-Bis[(phenyl-4-carbonate)methylene]carbonimidic Dihydrazide Hydrochloride. A solution of 4-formylphenylcarbonate (1.32 g, 8.8 mmol, 2.2 equiv) and 1,3-diaminoguanidine hydrochloride (500 mg, 4 mmol, 1 equiv) in ethanol (5 mL) was refluxed for 16 h. Diethyl ether (10 mL) was added, and the product carbonimidic dihydrazide crashed out of solution as a white solid. The product was filtered, washed with diethyl ether, and recrystallized from methanol as a hydrochloride salt. Yield: 600 mg, 39%. ^{1}H NMR (400 MHz, DMSO-d6) δ 13.13 (s, 2H), 12,57 (s, 2H), 8,70 (s, 2H), 8.52 (s, 2H), 8.11 (d, J = 7.7 Hz, 4H), 8.03 (d, J = 7.7 Hz, 4H). ^{13}C NMR (400 MHz, DMSO-d6) δ 193.48, 167.31, 167.02, 153.47, 148.46. 139.35, 137.72, 136.12, 132.79, 130.39, 130.03, 128.40. HRMS found 354.1195, M+H. MP = 336−338 °C.

30: 2,2′-Bis[(4-carbonamidophenyl)methylene]-carbonimidic Dihydrazide Hydrochloride. A solution of 4-carbonamidobenzaldehyde (655 mg, 4.4 mmol, 2.2 equiv) and 1,3-diaminoguanidine hydrochloride (250 mg, 2 mmol, 1 equiv) in ethanol (5 mL) was refluxed for 16 h. Diethyl ether (10 mL) was added, and the product carbonimidic dihydrazide crashed out of solution as a white solid. The product was filtered, washed with diethyl ether, and recrystallized from methanol as a hydrochloride salt. Yield: 770 mg, 100%. ^{1}H NMR (400 MHz, DMSO-d6) δ 12.43 (s, 2H), 8.85 (s, 2H), 8.50 (s, 2H), 8.13 (s, 2H), 8.04 (d, J = 7.8 Hz, 4H), 7.98 (d, J = 7.8 Hz, 4H), 7.50 (s, 2H). ^{13}C NMR (400 MHz, DMSO-d6) δ 167.70, 153.41, 148.59, 136.32, 136.26, 128.30, 128.15. HRMS found 352.5100, M+H. MP = 309−311 °C.

31: 2,2′-Bis[(4-sulphonamidophenyl)methylene]-carbonimidic Dihydrazide Hydrochloride. A solution of 4-sulphonamidobenzaldehyde (814 mg, 4.4 mmol, 2.2 equiv) and 1,3-diaminoguanidine hydrochloride (250 mg, 2 mmol, 1 equiv) in ethanol (5 mL) was refluxed for 16 h. Diethyl ether (10 mL) was added, and the product carbonimidic dihydrazide crashed out of solution as a white solid. The product was filtered, washed with diethyl ether, and recrystallized from methanol as a hydrochloride salt. Yield: 920 mg, 100%. ^{1}H NMR (400 MHz, DMSO-d6) δ 12.41, (s, 2H), 8.69 (s, 2H), 8.50 (s, 2H), 8.14 (6, J = 7.6 Hz, 4H), 7.91 (d, J = 7.6, 4H), 7.49 (s, 4H). ^{13}C NMR (400 MHz, DMSO-d6) δ 153.49, 146.00, 136.81, 128.72, 125.43. HRMS found 424.0852, M+H. MP = 285−287 °C.

32: 2,2′-Bis[(4-propynoxyphenyl)methylene]carbonimidic Dihydrazide Hydrochloride. A solution of 4-propynoxybenzaldehyde (704 mg, 4.4 mmol, 2.2 equiv) and 1,3-diaminoguanidine hydrochloride (250 g, 2 mmol, 1 equiv) in ethanol (5 mL) was refluxed for 16 h. Diethyl ether (10 mL) was added, and the product carbonimidic dihydrazide crashed out of solution as a white solid. The product was filtered, washed with diethyl ether, and recrystallized from methanol as a hydrochloride salt. Yield: 0.82 g, 100%. ^{1}H NMR (400 MHz, DMSO-d6) δ 12.19 (s, 2H), 8.42 (s, 2H), 8.37 (s, 2H), 7.91 (d, J = 8.4 Hz, 4H). ^{13}C NMR (400 MHz, DMSO-d6) δ 159.67, 153.09, 129.93, 127.06, 115.56, 79.38, 79.06, 56.09. HRMS found 374.1606, M+H. MP = 217−219 °C.

33: 2,2′-Bis[(4-morpholinophenyl)methylene]-carbonimidic Dihydrazide Hydrochloride. A solution of 4-morpholinobenzaldehyde (840 mg, 4.4 mmol, 2.2 equiv) and 1,3-diaminoguanidine hydrochloride (250 mg, 2 mmol, 1 equiv) in ethanol (5 mL) was refluxed for 16 h. Diethyl ether

(10 mL) was added, and the product carbonimidic dihydrazide crashed out of solution as a white solid. The product was filtered, washed with diethyl ether, and recrystallized from methanol as a hydrochloride salt. Yield: 850 mg, 90%. ^{1}H NMR (400 MHz, DMSO-d6) δ 11.84 (s, 2H), 8.26 (s, 4H), 7.77 (d, J = 8.1 Hz, 4H), 7.01 (d, J = 8.1 Hz, 4H), 3.75 (s, 8H), 3.24 (s, 8H). ^{13}C NMR (400 MHz, DMSO-d6) δ 153.09, 152.75, 149.18, 129.58, 123.87, 114.53, 66.40, 47.78. HRMS found 436.2449, M+H. MP = 280−282 °C.

34: 2,2′-Bis[(3-tetrazole-1-phenyl)methylene]-carbonimidic Dihydrazide Hydrochloride. A solution of 3-tetrazole-1-benzaldehyde (766 mg, 4.4 mmol, 2.2 equiv) and 1,3-diaminoguanidine hydrochloride (250 g, 2 mmol, 1 equiv) in ethanol (5 mL) was refluxed for 16 h. Diethyl ether (10 mL) was added, and the product carbonimidic dihydrazide crashed out of solution as a white solid. The product was filtered, washed with diethyl ether, and recrystallized from methanol as a hydrochloride salt. Yield: 870 mg, 100%. This compound was not sufficiently soluble in chromatography solvents to obtain a quantitative purity by HPLC. ^{1}H NMR (400 MHz, DMSO-d6) δ 8.84 (s, 2H), 8.75 (s, 2H), 8.66 (s, 2H), 8.20 (t, J = 9.0 Hz, 4H), 7.76 (t, J = 7.78, 2H). ^{13}C NMR (400 MHz, DMSO-d6) δ 152.27, 147.44, 133.85, 129.83, 129.27, 128.30, 125.70, 124.25. HRMS found 402.1641, M+H. MP = 289−291 °C.

35: 2,2′-Bis[(4-tetrazole-1-phenyl)methylene]-carbonimidic Dihydrazide Hydrochloride. A solution of 4-tetrazole-1-benzaldehyde (1.53 g, 8.8 mmol, 2.2 equiv) and 1,3-diaminoguanidine hydrochloride (500 mg, 4 mmol, 1 equiv) in ethanol (5 mL) was refluxed for 16 h. Diethyl ether (10 mL) was added, and the product carbonimidic dihydrazide crashed out of solution as a white solid. The product was filtered, washed with diethyl ether, and recrystallized from methanol as a hydrochloride salt. Yield: 1.75 g, 100%. This compound was not sufficiently soluble in chromatography solvents to obtain a quantitative purity by HPLC. ^{1}H NMR (400 MHz, DMSO-d6) δ 8.70 (s, 2H), 8.52 (s, 2H), 8.20 (s, 8H), 3.47−3.42 (m, 2H), 1.06 (t, J = 7.0 Hz, 2H). ^{13}C NMR (400 MHz, DMSO-d6) δ 153.39, 148.45, 136.23, 129.19, 127.77. HRMS found 402.1641, M+H. MP = 277−279 °C.

36: 2,2′-Bis{[4-(2,2-difluoro-2,3-dioxazolo)]phenyl}-methylene]carbonimidic Dihydrazide Hydrochloride. A solution of 4-(2,2-difluoro-2,3-dioxazolo)benzaldehyde (820 mg, 4.4 mmol, 2.2 equiv) and 1,3-diaminoguanidine hydrochloride (250 mg, 2 mmol, 1 equiv) in ethanol (5 mL) was refluxed for 16 h. Diethyl ether (10 mL) was added, and the product carbonimidic dihydrazide crashed out of solution as a white solid. The product was filtered, washed with diethyl ether, and recrystallized from methanol as a hydrochloride salt. Yield: 800 mg, 87%. ^{1}H NMR (400 MHz, DMSO-d6) δ 12.58 (s, 2H), 8.66 (s, 2H), 8.60 (s, 2H), 8.04 (d, J = 7.74 Hz, 2H), 7.52 (dd, J = 7.97, 0.93, 2H), 7.32 (t, J = 8.12, 2H). ^{13}C NMR (400 MHz, DMSO-d6) δ 142.55, 140.90, 140.27, 130.60, 123.91, 120.71, 116.23, 111.11. HRMS found 426.0814, M+H. MP = 276−278 °C.

37: 2,2′-Bis[(2,4-dimethoxy-5-chlorophenyl)ethylidene]-carbonimidic Dihydrazide Hydrochloride. A solution of 2,4-dimethoxy-5-chloroacetophenone (655 mg, 4.4 mmol, 2.2 equiv) and 1,3-diaminoguanidine hydrochloride (250 mg, 2 mmol, 1 equiv) in ethanol (5 mL) was refluxed for 16 h. Diethyl ether (10 mL) was added, and the product carbonimidic dihydrazide crashed out of solution as a white solid. The product was filtered, washed with diethyl ether, and recrystallized from methanol as a hydrochloride salt. Yield: 400

mg, 39%. ^{1}H NMR (400 MHz, DMSO-d6) δ 11.64 (s, 2H), 8.55 (s, 2H), 7.68 (s, 2H), 6.85 (s, 2H), 3.94 (s, 6H), 3.91 (s, 6H), 2.31 (s, 6H). ^{13}C NMR (400 MHz, DMSO-d6) δ 157.97, 156.84, 154.64, 154.53, 130.97, 130.65, 120.76, 112.75, 98.42, 98.30, 57.21, 56.96, 56.79, 32.07, 19.31. HRMS found 482.1350, M+H. MP = 229−231 °C.

38: 2-(Phenylmethylene)-2′-[(2-Fluorophenyl)methylene]-carbonimidic Dihydrazide Hydrochloride. A solution of benzaldehyde (466 mg, 4.4 mmol, 1.1 equiv), 2-fluorobenzaldehyde (546 mg, 4.4 mmol, 1.1 equiv), and 1,3-diaminoguanidine hydrochloride (500 mg, 4 mmol, 1 equiv) in ethanol (5 mL) was refluxed for 16 h. Diethyl ether (10 mL) was added, and the product carbonimidic dihydrazide crashed out of solution as a white solid. The product was filtered, washed with diethyl ether, and recrystallized from methanol as a hydrochloride salt. This product was isolated as a mixture also containing **24** and **2**. **38** was the dominant product accounting for greater than 50% of the total material in the mixture. Yield: 1.27 g mixture, 50%. ^{1}H NMR (400 MHz, DMSO-d6) δ 12.30 (s, 3H), 8.69 (s, 1H), 8.62 (s, 1H), 8.57 (s, 1H), 8.53 (s, 1H), 8.44 (s, 1H), 8.35 (t, J = 6.5 Hz, 1H), 7.96−7.94 (m, 2H), 7.56 (q, J = 8.1 Hz, 1H), 7.51−7.49 (m, 2H), 7.34 (t, J = 8.1, 2H). ^{13}C NMR (400 MHz, DMSO-d6) δ 133.27, 130.78, 128.74, 127.89, 127.28, 124.80, 115.88. HRMS found 284.1301, M+H. MP = 281−283 °C.

General Biology: Parasite Culture and Drug Sensitivity. The following parasite strains were used in this study and obtained through BEI Resources, NIAID, NIH. *Plasmodium falciparum*, Strain D6 (MRA-285, originally from Sierra Leone, has modest resistance to mefloquine).[46] Strain Dd2 (MRA-150, originated from Indochina; derived from W2-mef and is resistant to chloroquine, pyrimethamine, and mefloquine). *P. falciparum* strain Tm90−C2B (Thailand; resistant to mefloquine, chloroquine, atovaquone, and pyrimethamine) was obtained directly from the Division of Experimental Therapeutics of Walter Reed Army Institute of Research (WRAIR) in Silver Spring, Maryland, United States.[39] Strain SB1-A6 (MRA-1002, Sierra Leone was derived from D6 clone and is resistant to Atovaquone and ELQ-300.[40]

P. falciparum parasites were thawed from frozen stock and cultured in suspended human erythrocytes (Lampire Biological Laboratories, Pipersville, PA) not more than 28 days old at 2% hematocrit. The culture medium used was RPMI-1640, supplemented with 25 mM HEPES buffer, 25 mg/L gentamicin sulfate, 45 mg/L hypoxanthine, 10 mM glucose, 2 mM glutamine, and 0.5% Albumax II (complete medium).[43] Cultures were maintained in a standard low oxygen atmosphere (5% O_2, 5% CO_2, 90% N_2) in an environmental chamber and incubated at 37 °C. Cultures were subpassaged every 3−4 days into a fresh culture flask containing complete media and erythrocytes.

IC_{50} Determination by the Fluorescence-Based SYBR Green Assay. The aminoguanidine series was assessed for *in vitro* antiplasmodial activity using the fluorescence-based SYBR Green assay described previously by Smilkstein and co-workers.[38] Compounds were evaluated in quadruplicate in flat-bottomed Costar clear 96-well plates (Model #3585). A 2-fold serial dilution of each compound was performed across the columns of the test plates starting with 20 μM and ending with a final untreated column to serve as control wells. Asynchronous parasite infected erythrocytes in growth media were added to each well for a total volume of 100 μL, final hematocrit of 2%, and initial parasitemia of 0.2%. The

commercial malaria drugs atovaquone and chloroquine were used as control drugs. Test plates were incubated for 72 h at 37 °C in an environmental chamber with a controlled low oxygen atmosphere (5% O_2, 5% CO_2, 90% N_2). After the incubation period, 100 μL SYBR Green I dye-detergent solution was added to each well, and the plates were incubated at ambient temperature and atmosphere in the dark for at least 1 h. Fluorescence was read at 497 nm excitation and 520 nm emission bands using a Spectramax iD3 plate reader. Fluorescence readings were normalized with respect to the untreated control wells representing normal parasite growth and plotted against the logarithm of drug concentration. An IC_{50} was determined for each compound by fitting this data to a variable slope nonlinear regression curve using Graphpad Prism software (v. 8).

HepG2 Cytotoxicity Assay. Compounds were prepared as 10 mM stock solutions in DMSO. Human hepatocarcinoma (HepG2) cells were maintained in culture at 37 °C in a humidified 5% CO_2 atmosphere in RPMI-1640 medium containing 10% fetal bovine serum. HepG2 cells were added to each well of flat bottom 96-well tissue culture plates at an initial density of 2×10^4 cells per well and an initial volume of 160 μL complete medium per well. After an overnight incubation at 37 °C to adhere the cells to the culture plates, 40 μL drug solutions in complete medium were applied to each well at a final concentration range of 0 to 200 μM across each plate. Drugs were tested in triplicate or quadruplicate. The cells were incubated for 24 h at 37 °C and 5% CO_2 with the drug solutions, which were then aspirated and replaced with 200 μL per well of complete medium for an additional 24 h incubation under the same conditions. To each well was added 20 μL of resazurin (Alamar Blue) in PBS buffer to a final concentration of 10 μM, and the plates were incubated for 3 h. Fluorescence was measured at 560 nm excitation and 590 nm emission bands using a Spectramax iD3 plate reader. Fluorescence readings were normalized with respect to the untreated control wells and plotted against the logarithm of drug concentration. An IC_{50} was determined for each compound by fitting this data to a variable slope nonlinear regression curve using Graphpad Prism software (v. 8).

***In Vivo* Efficacy against Murine Malaria.** The *in vivo* ED_{50} and ED_{90} of selected aminoguanidines was measured using a modified 4-day Peters test. Female CF1 mice from Charles River Laboratories were inoculated intravenously with approximately $2.5−5.0 \times 10^4$ parasitized erythrocytes (murine malaria *P. yoelii*, Kenya strain MR4 MRA-428) from a donor mouse (experiment day zero). On the following 4 days (experiment days 1−4), solutions of the test compounds in PEG-300 (or PEG-300 only for control mice) were administered by oral gavage once daily. Aminoguanidines were initially assessed at 2.5, 5, and 10 mg/kg/day, and experiments were repeated to adjust the dose range as needed to obtain an interpolated ED_{50} and ED_{90} value (**1** required a dosing down to 0.1 mg/kg/day to attain this result). Experiments were repeated with doses up to 25 mg/kg/day to obtain a nonrecrudescence dose, though only **16** was found to produce a cure in this model. Parasitemia of each mouse was determined by microscopic examination of Giemsa stained blood smears on day 5. ED_{50} and ED_{90} values were assessed by generating dose−response curves relative to untreated controls using Graphpad Prism (v. 8). Mice were considered cured of malarial infection if they maintained 0% parasitemia at experiment day 30. The procedures involved, together with

all matters relating to the care, handling, and housing of the animals used in this study, were approved by the Portland VA Medical Center Institutional Animal Care and Use Committee.

Murine Microsomal Stability. Metabolic stability studies of selected aminoguanidines were performed at ChemPartner, Shanghai, China. Compounds were incubated at 37 °C and 1 μM concentration in murine liver microsomes (Corning) for 1 h at a protein concentration of 0.5 mg/mL in potassium phosphate buffer at pH 7.4 containing 1.0 mM EDTA. The metabolic reaction was initiated by NADPH and quenched with ice-cold acetonitrile at 15 min increments up to 1 h. The progress of compound metabolism was followed by LC-MS/MS (ESI positive ion, LC-MS/MS-034(API-6500+) using a C18 stationary phase (ACQUITY UPLC BEH C18 (2.1 × 50 mm, 1.7 μm)) and a MeOH/water mobile phase containing 0.25% FA and 1 mM NH$_4$OAc. Imipramine or Osalmid were used as internal standards, and ketanserin was used as a metabolically unstable control compound. Concentration versus time data for each compound were fitted to an exponential decay function to determine the first-order rate constant for substrate depletion, which was then used to calculate the degradation half-life ($t_{1/2}$) and predicted intrinsic clearance value (Cl$_{int}$) from an assumed murine hepatic blood flow of 90 mL/min/kg.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsinfecdis.1c00001.

Calculated error values for antiplasmodial IC$_{50}$ and cytotoxicity assays (PDF)

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Notes

The authors declare the following competing financial interest(s): Coauthors M.R. and A.K. have filed an invention disclosure relating to selected compounds described in this paper to the United States Department of Veterans Affairs and to Oregon Health & Science University as required by law.

■ DEDICATION

This manuscript is dedicated to Dr. Jonathan Vennerstrom in honor of his 65th birthday and for his outstanding contributions to the field of antimalarial drug research and development.

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