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NOTE

Antimalarial activity of kinase inhibitor, nilotinib, *in vitro* and *in vivo*

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Malaria has been historically recognized as one of the world's most devastating diseases. The World Health Organization now recommends Artemisinin-based Combination Therapies (ACTs) for treatment of malaria. However, it has been reported that the parasite sensitivity against ACTs is reduced significantly by the spread of parasites that have developed resistance to other antimalarial drugs, such as mefloquine and lumefantrine.[1] Furthermore, it was reported that a molecular marker of artemisinin-resistant *Plasmodium falciparum,* PF3D7_1343700 kelch propeller domain (K-13-propeller), is associated with artemisinin resistance in parasites isolated in western Cambodia, where resistance to virtually all the known antimalarial drugs has been reported.[2] Although parasite resistance to artemisinin has been detected only in South-East Asian countries, development of new antimalarial drugs, especially those having novel modes of action, is urgently required.

During antimalarial drug development around the world, it has been reported that several kinase inhibitors (staurosporine, cyclin-dependent kinase inhibitors, etc.) show moderate (or weak) *in vitro* antimalarial effects against erythrocytic stage parasites, and targets predicted to be related to kinases involved in the *Plasmodium* erythrocyte stage cycle, although the actual targets remain unknown.[3–7] In addition, *P. falciparum* kinases PfPK5, Pfmrk and PfPKA-C, regulating nuclear division in the erythrocytic stage parasite, were identified as potential drug targets and inhibitors are under development for innovating new antimalarial drugs.[8–12] It was reported that there are 65 malaria eukaryotic protein kinases (ePKs) but no malaria kinases are classified into the tyrosine kinase (TK) group and sterile phenotype kinase group based on the analysis using several bioinformatics tools.[13] Furthermore, it has been suggested that the *Plasmodium* parasite kinome is the most druggable, targeting not only erythrocytic stage parasite (asexual and sexual) but also the insect-dwelling and liver stages of the parasite.[14,15] A recent paper also indicated that 36 ePKs in the *Plasmodium* parasite kinome were essential for homeostasis in the *P. falciparum* erythrocytic asexual stage and also in the rodent malaria parasite (*P. berghei*) orthologous from the phospho-proteomic analysis.[16] However, there are no antimalarial drugs targeting *Plasmodium* kinases so far. In this paper, we report an evaluation of the antimalarial activity of clinically-used seven kinase inhibitor drugs against erythrocytic asexual stage parasites, as well as cytotoxicity against MRC-5 cells, to help determine their potential for antimalarial drugs. We found that nilotinib, approved for the treatment of chronic myeloid leukemia targeting Bcr-Abl TK, demonstrates *in vitro* antimalarial activity against erythrocytic asexual parasite stages. *In vivo* efficacy of nilotinib was also observed using a rodent malaria model.

The evaluation of antimalarial activity and cytotoxicity were conducted as previously reported.[17] Briefly, cultured *P. falciparum* (chloroquine sensitive FCR3 strain and chloroquine resistant K1 strain) in Type A+ blood were seeded in 96-well culture plates (parasitaemia 0.5–1%, Hematocrit 2.0%) and incubated with test drugs for 72 h. After incubation, parasite lactate dehydrogenase activity was assayed to determine parasite growth and calculate the anti-malarial activity in comparison with the controls that had received no drugs. Cytotoxicity was tested against MRC-5 cells. Cultured MRC-5 cells were seeded in 96-well culture plates with 1×10^3 cells per well and test drugs were added to the each well on the following day. After 7 days of incubation, cell proliferation was determined by MTT assay allowing a calculation of cytotoxicity. Selectivity indexes (SIs) were calculated by the following formula: SI = IC_{50} for cytotoxicity/IC_{50} for antimalarial activity. In the *in vivo* evaluation, *P. berghei* N strain was administered into ICR (CD1) mice (dose of 2×10^6 parasites). First treatment was started 2 h post-infection (D0) and drug was administered every 24 h for 3 days (D1–3). Parasitaemia was determined on day 4 (D4) with blood smears to calculate the percentage inhibition.

All kinase inhibitors were obtained commercially in the form of clinically-used salts (Figure 1). Table 1 shows *in vitro* antimalarial activity, cytotoxicity against MRC-5 cells and SI of commercially available kinase inhibitor drugs (sunitinib, sorafenib, erlotinib, lapatinib, dasatinib, imatinib and nilotinib),[18] along with two known antimalarial drugs chloroquine and artesunate. Among them, sunitinib, a multi-TK inhibitor, showed the most potent antimalarial activity (IC_{50}: 0.11–0.15 μg ml^{-1}), but it also displayed moderate cytotoxicity against MRC-5 cells (IC_{50}: 3.19 μg ml^{-1}). Sorafenib also known to inhibit several TKs in addition to its primary target B-Raf

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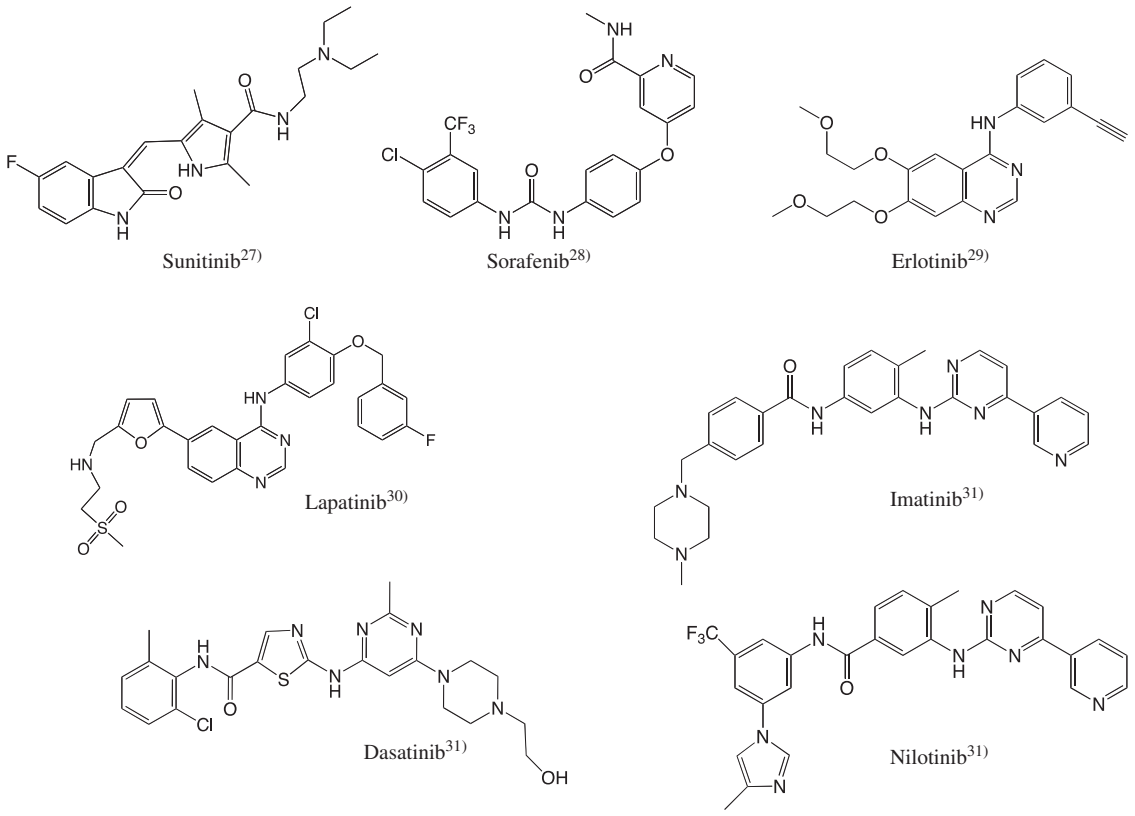

Figure 1 Structures of kinase inhibitors.[27–31]

Table 1 ***In vitro*** **antimalarial activity, cytotoxicity and SIs of kinase inhibitors**

		IC_{50}					
		Antimalarial activity			*Cytotoxicity*[a]		
		PfK1		*PfFCR3*	*MRC-5 cells*	*SI*[b]	
Compound	*Target kinase*	*μg ml*$^{-1}$ *(μ/M)*	*SD*	*μg ml*$^{-1}$ *(μ/M)*	*μg ml*$^{-1}$ *(μ/M)*	*MRC-5/pfK1*	*MRC-5/pfFCR3*
Sunitinib	Multi	0.15 (0.28)	±0.01[c]	0.11 (0.21)	3.19 (5.99)	21.3	29
Sorafenib	B-Raf+Multi	8.66 (13.59)	ND	ND	>100.00 (>156.98)	>11.5	—
Erlotinib	TK (EGFR)	>12.50 (>29.08)	ND	ND	ND	—	—
Lapatinib	TK (EGFR/HER2)	1.66 (1.76)	ND	ND	4.4 (4.66)	2.7	—
Dasatinib	Bcr-Abl+Src family TK	8.57 (16.94)	ND	ND	13.65 (26.98)	1.6	—
Imatinib	Bcr-Abl TK	8.02 (13.6)	ND	ND	14.5 (24.59)	1.8	—
Nilotinib	Bcr-Abl TK	1.22 (2.09)	±0.02[c]	0.64 (1.01)	89.07 (152.52)	73	139.2
Chloroquine[d]		301.09 (584.43)	±4.67	19.78 (38.41)	18 570 (36 045)	61.7	938.8
Artesunate[d]		2.85 (7.41)	±0.1	1.84 (4.78)	15 040 (39 123)	5200	12 800

Abbreviations: ND, not determined; *Pf*FCR3, chloroquine sensitive *P. falciparum* FCR3 strain; *Pf*K1, chloroquine resistant *P. falciparum* K1 strain; SD, standard deviation; SI, selectivity index.
[a]Compounds with antimalarial (K1) IC_{50} value of $<12.5\,\mu g\,ml^{-1}$ were subjected to cytotoxicity evaluation ($n=1$) using an MRC-5 cell line (normal human fetal lung fibroblast cells).
[b]SI = IC_{50} for cytotoxicity/IC_{50} for antimalarial activity.
[c]Compounds showing $<1.56\,\mu g\,ml^{-1}$ IC_{50} value against *Pf*K1 in the first screening ($n=1$) were repeatedly experimented ($n=3$) and also subjected to the anti-*Pf*FCR3 activity assay ($n=1$) to see whether they exhibited the results depending on the strain.
[d]IC_{50} values were shown by ng ml^{-1} and nM.

kinase, a serine-threonine kinase, was moderately active against malaria parasites, with an IC_{50} value of $8.66\,\mu g\,ml^{-1}$. Erlotinib and lapatinib are TK inhibitors specific to epidermal growth factor receptor (EGFR) or dual EGFR/HER2 receptors, respectively. Interestingly, erlotinib did not show any antimalarial activity below a concentration of $12.5\,\mu g\,ml^{-1}$, but lapatinib exhibited a moderate antimalarial activity, with an IC_{50} value of $1.66\,\mu g\,ml^{-1}$. Lapatinib is known to inhibit EGFR/HER2 receptors with very slow off-rate by inducing conformational change of kinases,[19] suggesting that some kinases in the *Plasmodium* parasite kinome may exist that are sensitive to such compounds. The cytotoxicity (IC_{50}) of lapatinib was $4.4\,\mu g\,ml^{-1}$ with a low SI of 2.7. Dasatinib, imatinib and nilotinib

Table 2 ***In vivo*** **antimalarial activity of nilotinib**

Compound	Dosage		Route	Inhibition (%)
	mg kg^{-1} day^{-1}	*Duration*		
Nilotinib	30	4 days	i.p.	36.3
	60			83.3
	30	4 days	p.o.	23.9
	60			75.8
Artesunate	10	4 days	i.p.	86.7

are all TK inhibitors targeting the Bcr-Abl kinase. Imatinib showed moderate antimalarial activity (IC_{50} value of 8.02 μg ml^{-1}) but its SI was 1.8, because of the relatively low IC_{50} value against MRC-5 (14.50 μg ml^{-1}). Dasatinib also inhibits other TKs belonging to the Src family, in addition to Bcr-Abl kinase, but it exhibits a similar potency and SI to imanitib, indicating that no kinases exist in the *Plasmodium* parasite kinome that are structurally similar to the Src family kinases. Niloitnib was a potent inhibitor against both the drug resistant strain (K1) and drug sensitive strain (FCR3), with IC_{50} values of 1.22 and 0.64 μg ml^{-1}, respectively, coupled with a significantly safe profile (SI: 73.0 and 139.2, respectively). These results imply the possible existence of malaria kinases having a binding site structurally similar to that of human Abl kinase. To evaluate the antimalarial activity of nilotinib, we investigated *in vivo* antimalarial activity using a mouse model. As shown in Table 2, nilotinib displayed drug potency with 36.3% and 83.3% parasite growth inhibition in *in vivo* antimalarial evaluation when the mice were treated with nilotinib intraperitoneally (30 and 60 mg kg^{-1} × 4 i.p.). It was noteworthy that oral treatment with nilotinib also dose-dependently inhibited the parasite growth (23.9% and 75.8% inhibition at 30 and 60 mg kg^{-1} × 4, p.o. respectively). The efficacy of nilotinib at 60 mg kg^{-1} × 4 oral treatment was comparable to that of artesunate, one of the most important clinically-used antimalarial drugs, with 10 mg kg^{-1} × 4 i.p. treatment.

As there are no TKs in the *Plasmodium* kinome,[13] the observed antimalarial activity of nilotinib should not be via TK inhibition. The fact that other Abl kinase inhibitors, dasatinib and imatinib, also showed antimalarial activity, suggests a possibility of the existence of malaria kinases which has a pocket that is structurally similar to the ATP-binding site of human Abl kinase, although the antimalarial target of nilotinib is unknown. The core scaffold of nilotinib, a phenylaminopyrimidine, is a common substructure seen in ATP-competitive kinase inhibitors. Imatinib, having the same core scaffold as nilotinib, showed *in vitro* antimalarial activity. There are also a few reports concerning ATP-competitive inhibitors of *P. falciparum* kinases, including PfPK5, PFPK7 and *pf*CDPK4.[20–22] Therefore, the molecular target of nilotinib might be one of the plasmodial kinases having an ATP-binding site similar to that of human Abl kinase.

Another plausible target is the oxidoreductase family. Rix *et al.*[23] reported chemical proteomic analysis of Bcr-Abl inhibitors, imatinib, nilotinib and dasatinib, using K562 cells, and that imatinib and nilotinib were bound to the non-kinase target, oxidoreductase NQO2 (also known as QR2). This oxidoreductase was also identified as a novel target of the quinoline antimalarial chloroquine by Graves *et al.*[24]

Although *in vitro* antimalarial activity of nilotinib was 271–581 times weaker than that of artesunate, *in vivo* antimalarial activity of nilotinib with 60 mg kg^{-1} × 4 was as effective as artesunate (using 10 mg kg^{-1} × 4). This result could imply that nilotinib has a better bioavailability, moreover it has about 24–48 times longer serum half-life, compared with artesunate or its active metabolite, dihydroartemisinin, in humans when administered orally.[25,26]

In conclusion, this paper identifies the *in vitro* and *in vivo* antimalarial activity of nilotinib, and these findings suggested that further investigation is needed to evaluate the possibility of using nilotinib for developing new antimalarials. Investigation of its mode of action is also currently under way.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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