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Anti-malarial activity of HCl salt of SKM13 (SKM13-2HCl)

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ABSTRACT

Malaria is among the most devastating and widespread tropical parasitic diseases in developing countries. To prevent a potential public health emergency, there is an urgent need for new antimalarial drugs, with single-dose cures, broad therapeutic potential, and novel mechanism of action. We synthesized HCl salt of SKM13 (SKM13-2HCl) based on the modification of SKM13 to improve solubility in water. The anti-malarial activity of the synthesized drug was evaluated in both *in vitro* and *in vivo* models. The selective index indicated that SKM13-2HCl showed the same effectiveness with SKM13 in *Plasmodium falciparum* in in-vitro. Even though, *in vivo* mouse study demonstrated that SKM13 (20 mg/kg) at single dose could not completely inhibit *P. berghei* growth in blood. The survival rate increased from 33 to 90% at 15 days after infection. However, SKM13-2HCl (20 mg/kg) at a single dose increased the survival rate up to 100% at the same duration. Ultra-High-Performance Liquid Chromatography (UHPLC) showed that solubility in water of SKM13 and SKM13-2HCL was 0.389 mg/mL and 417 mg/mL, respectively. Pharmacokinetics (PK) analysis corresponded to the increased solubility of SKM13-2HCl over SKM13. Haematological parameters [red blood cell (RBC) count, haemoglobin level, and haematocrit level] supported the comparable efficacy of SKM13 and SKM13-2HCl in a 4-day suppression test. One mode of these drugs was found to be activating phosphorylation of eIF2α, hallmark of ER-stress, to kill parasite. Novel salt derivative of SKM13 (SKM13-2HCl) have enhanced anti-malarial activity against *P. falciparum* with endoplasmic reticulum (ER)-stress and salt form of SKM13 is an excellent direction to develop anti-malarial drug candidate in mice model.

1. Introduction

Malaria is one of the world's greatest unmet medical needs, that leads to the death of around half a million patients per year, mostly pediatric patients in tropical low economy communities.

The treatment of malaria in such communities is challenging due to supply and adherence issues.

It has been shown that organic acid salt forms of basic drugs, such as amines, have higher aqueous solubilities than their corresponding halide salts (Agharkar et al., 1976). This technique has found important application in the development of more soluble salt forms of drugs to improve their bioavailability and ease in formulation.

Efforts to develop orally administered drugs tend to place an exceptional focus on aqueous solubility as this is an essential criterion for their absorption in the gastrointestinal tract.

It also is a commonly applied technique to rectify suboptimal drug properties, such as poor stability, physical quality, and purity, optimize process chemistry, reduce toxicity, alter absorption in the

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gastrointestinal tract, and enhance organoleptic palatability.

Pharmacological intervention is still regarded as a key strategy to address malaria as a disease, with a strong push for new candidate drugs to which the parasite has no or limited resistance and with sufficient potency and favorable pharmacokinetic profiles to enable a single dose treatment (Mischlinger et al., 2016). So, improvement of salt form was reported in other reports. An analysis of the U.S. Food and Drug Administration (FDA) Orange Book database revealed that half of the approved active pharmaceutical ingredients (APIs) are salt forms (Paulekuhn et al., 2007).

In today's pharmaceutical development paradigm, salt screening is implemented early in research with the aims of improving developability and maximizing *in vivo* exposure from orally administered solid dosage forms (Korn and Balbach, 2014). For many poorly soluble compounds, adequate exposure could not be achieved without converting them to more soluble salt forms. Salt forms with higher solubility are also often selected to develop parenteral products to prevent precipitation at the injection sites because precipitated drugs can cause adverse effects (Kalepu and Nekkanti, 2015).

So far, there were several efforts to improve anti-malaria drug's solubility by inconvenient modification using multifluid nozzle spray containing poorly water-soluble anti-malarial drug (Date et al., 2007; Sahoo et al., 2009; Puttappa et al., 2019).

In previous study, we found that SKM13 was effective as anti-malarial candidate drugs but we noticed that there was a phenomenon that mouse looked like uncomfortable with intravenous injection of a solution of SKM13 in DMSO at high concentration (Yeo et al., 2017). Therefore, there were concerns that during intravenous injection, it was not directly distributed to body fluids and blood, but would be partially crystallized at the injection site, which decreased the actual pharmacological effect of SKM13. HCl salt of SKM13 was prepared to remove the possibility of experimental error due to a low solubility in water.

Therefore, in this study, SKM13 salt was developed to improve solubility because salt formation is one of the primary approaches to improve the developability of ionizable poorly water-soluble compounds (He et al., 2017).

However, antimalaria drug resistant is also a huge obstacle in malaria treatment, therefore this has necessitated the effort for seeking for a new drug which have effective activity on the drug resistant parasites. Therefore, in this study, we also perform the *in vitro* evaluation for SKM13 and SKM13-2HCl on the CQ-resistant parasite strain.

2. Materials and methods

2.1. Reagents, cells and parasites

Chloroquine (CQ), Atovaquine, and Dihydroartemisinin (DHA) were from Sigma-Aldrich (St. Louis, MO, USA). The Differential Quik III Stain Kit was obtained from Polysciences Inc. (Valley Road Warrington, PA, USA). Propidium iodide (PI) and SYTOX Green solution were brought from BD Pharmingen, Inc. (San Diego, CA, USA) and Life Technologies (Grand Island, NY, USA), respectively. The CQ-sensitive strain *Plasmodium falciparum* (3D7) (American Type Culture Collection, ATCC PRA-405D) and Vero cells were obtained from American Type Culture Collection (ATCC) (Manassas, VA, USA). The Atovaquone-resistant rodent malaria strain *Plasmodium berghei* NAT (MRA-415), was obtained from BEI Resources (Manassas, VA, USA).

2.2. Synthesis of SKM13-2HCl

SKM13-2HCl was synthesized following the scheme presented in Fig. 1 and SKM13 was prepared as previously described (Yeo et al., 2017). The chemical structures of each products during synthesis were confirmed using proton NMR. NMR data is described in Table S1.

2.3. In vitro cytotoxicity and anti-malarial activity study

To evaluate the cytotoxicity of SKM13-2HCl with mammalian cell, Vero cell (Kidney epithelial cells extracted from a normal adult African green monkey (Chlorocebus sp.)) were pre-seeded in a 96-well plate 12h prior to inoculation of serially diluted of control drugs (CQ, DHA, SKM13), and test drug SKM13-2HCl for 48h. And therefore, cells were then inoculated with 20 μL of CellTiter 96® AQueous One Solution reagent (Madison, USA). Finally, the inoculated plates were measured at the absorbance at 490 nm using a microplate reader from Biotex (Agilent Technologies., Santa Clara, CA, USA). The results were collected and calculated for 50% cell cytotoxicity (CC_{50}) using Prism 9.0 (GraphPad, La Jolla, CA, USA).

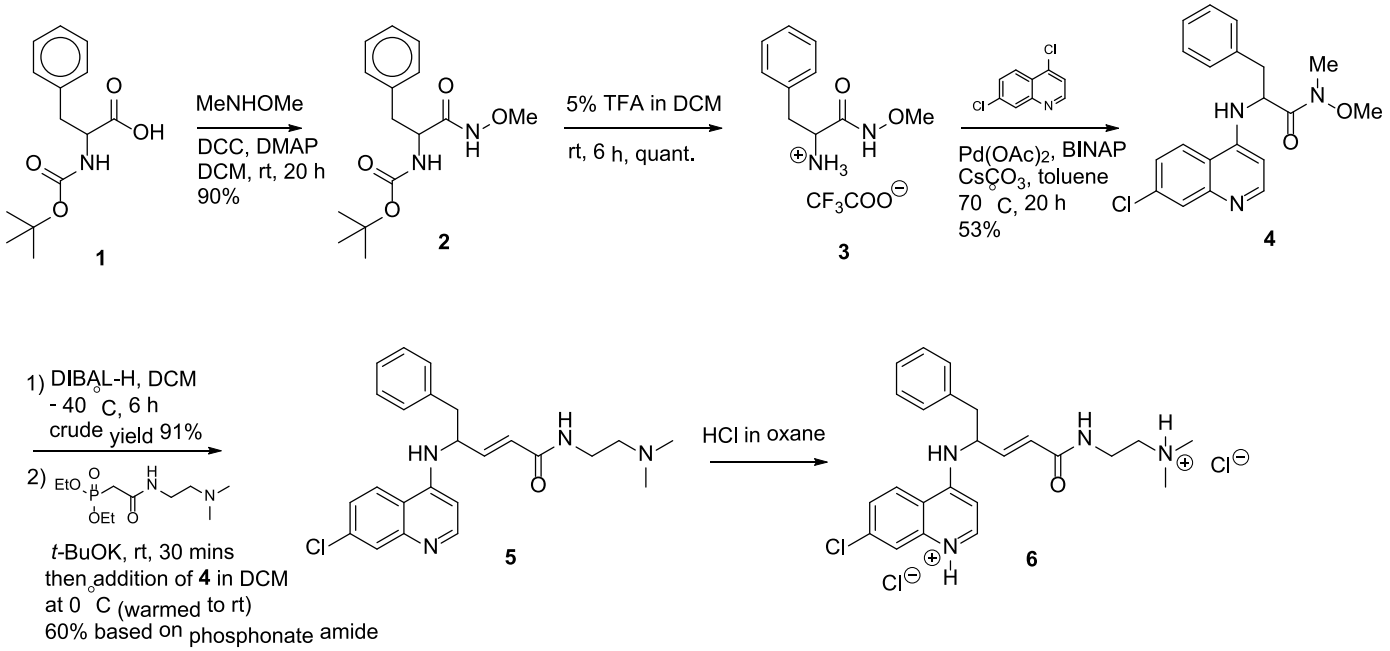

Fig. 1. Synthesis scheme of the new antimalarial derivative (SKM13-2HCl).

To explore the inhibitory effect of SKM13-2HCl on malarial growth *in vitro*, Chloroquine susceptibility malaria strain *Plasmodium falciparum* 3D7 was used in this study. Parasites were propagated in 5% hematocrit of O+ human Red Blood Cells (RBCs) as previously described (Yeo et al., 2017), suspended in RPMI 1640, supplemented with L-Glutamine, 25 mM HEPES, 1% AlbuMAX™ILipid-Rich BSA, 25 mM Sodium bicarbonate, 25 μg mL-1 gentamycin, 0.05% hypoxanthine, and 1% penicillin, and incubated at 37 °C in an atmosphere of 5% O2, 5% CO2, and 90% N2. Medium was changed daily with monitoring of parasitemia which was determined as the percentage of infected erythrocytes per a total of one thousand erythrocytes.

For *in vitro* inhibition of malaria growth, parasites were seeded in 48-well cell culture plate at 1% parasitemia, 2% hematocrit and incubated with serially diluted drugs for 48 h. After incubation time, 1 μL of the blood pellet from each well was mixed with 5 mM SYTOX green solution to obtain a final volume of 1 mL. The mixtures were incubated in the dark for 30 min at room temperature and the parasites infected erythrocyte populations were determined using BD FACSCanto™ Clinical Flow Cytometry System (BD Biosciences, Carlsbad, California, USA). Anti-malarial concentrations of the test drugs were determined as the half-maximal inhibitory concentration on malaria growth (IC_{50}), expressed as mean ± SEM of the three separate experiments performed in triplicate.

2.4. Study on inhibition of malaria growth in rodent model

Seven-week-old female ICR-1 mice were purchased from Orient (Orient BO. CO., Seoungnam, Korea) then housed at Wonkwang University under constant conditions with temperatures of ~18–23 °C and 40–60% humidity during the experiment. Animal experiments were performed using the experimental protocol approved by the Institutional Animal Care and Use Committee (IACUC) at Wonkwang University (WKU22-21).

The atovaquone-resistant strain *P. berghei* NAT was used to set up an infected mouse model. The parasites kept in liquid nitrogen were thawed at 37 °C and activated by maintaining via the serial passages of blood from mouse to mouse. ICR-1 mice were divided into 6 mice per group, except the normal control no parasites infected group, each mouse was injected with 10^7 *P. berghei* infected erythrocytes/100 μL by intraperitoneal (i.p.) injection. At one day post-infection (dpi), drug treatment with SKM13 (at 10, or 20, or 30 mg/kg) or SKM13-2HCl (at 10, or 20, or 30 mg/kg) was commenced and follow for 4 days post initial infection day. Dihydroartemisinin (DHA) (10 mg/kg), Atovaquone (4 mg/kg), and chloroquine (10 mg/kg) were intravenously (i.v.) administered once a day for four consecutive days as the control drug. Each day, tail blood was collected for FACS assessment of parasitaemia. Fifteen days following the infection, mouse weight and survival was checked every day, and hematology assays were conducted at 3, 6, 9, and 12 days post infection.

2.5. Hematological analysis

The hematology assays were performed using a fully automated blood cell counter LC-660 (HORIBA Medical, Seoul, South Korea). This assay evaluated White Blood Cell count (WBCs), Red Blood Cell count (RBCs), Haemoglobin (Hgb), and hematocrit (HCT).

2.6. In vivo PK properties in mice

To analyze PK properties, ICR mice were provided by Orient, South of Korea as previously described (Li et al., 2022) and kept at an institutional animal facility under specific-pathogen-free (SPF) conditions with temperatures of ~18–23 °C and 40–60% humidity during the experiment. They were fasted overnight and allowed free access to water before administration. All procedures involving animals were following the Regulations of Experiment Animal Administration issued by the Wonkwang University (WKU22-46). Drugs was dissolved with DMSO (Tween20) and DW as a stock solution (1.5 mg/mL, oral). The stock solution was orally administrated to three mice at the dose of 5 mg/kg at a single dose. Blood was collected at 0.25, 0.5, 1, 2, 4, 8, and 24 h after dosing for the oral group. About 500 μL blood samples were collected into K2-EDTA tubes and then immediately centrifuged at 8000 rpm per min for 6 min. The plasma obtained was stored at −80 °C until analysis. Plasma concentration of each compound was plotted to generate a regression trend line for each of the kinetics using PKSolver 2.0 (Zhang et al., 2010) and PK was shown as non-compartment analysis (NCA) by WOOJUNG BIO, Inc. (Suwon, Korea) based on LC/MS/MS spectrum of standard.

2.7. Analysis of cell lysates by Western blotting of eIF2a

Trophozoite parasite cultures at 5% haematocrit and 5% parasitemia were cultured in 6 wells cell culture plates 10h to 12 h before subjecting to the drug treatments at 37 °C. Concentrations used do not result in loss of binding of a nucleic acid probe within the 90 min but it would be sublethal if the parasites were maintained in the presence of drug through to the next asexual cycle.

RBC cytoplasmic contents were released with 0.15% (w/v) saponin and parasite pellets were washed with PBS. Parasite pellets were solubilized by RIPA buffer (Thermo Fisher) and mixed in reducing SDS-PAGE sample buffer, boiled at 95 °C for 5 min, resolved by SDS-PAGE on 10% Bis-Tris acrylamide gel. After gel electrophoresis, proteins on gels were transferred onto FVDF membrane by Mini Trans-Blot® Cell system (BIORAD) for 2 h 30 min in cold condition. Membranes were blocked with 5% (w/v) Bovine Serum Albumin (BSA) for 1 h at room temperature and probed with primary antibody overnight at 4 °C, followed by secondary antibody for 1 h at room temperature. Rabbit anti-phospho-eIF2α (Cell Signaling Technology-119A11; 1:1000); Rabbit anti-eIF2α (Cell Signaling Technology-D7D3; 1:3000); mouse anti-PfEF1α-3A10 (Made in our lab; 5 μg/mL) were used as primary antibodies diluted in 3% BSA. Secondary antibodies: goat anti-rabbit IgG-peroxidase (Cell signaling Technology; 1:3000), rabbit poly-antibody to mouse IgG-peroxidase (Abcam-Ab97046; 1:10,000). Clarity Western ECL substrate (Bio-Rad, Hercules, CA, USA) was detected using the ChemiDocTM XRS + Imaging System (Bio-Rad), expose automatically by machine imaging system. Biological replicates of some blots are provided in Supplementary Fig. S4. Uncropped versions of some blots are provided in Supplementary Fig. S5.

2.8. Statistical analysis

Data were presented as mean ± standard (SD) deviations of the mean ± SD of biological replicates and plotted using GraphPad Prism 9.0 (GraphPad, La Jolla, CA, USA). The experiments were compared by performing two-way analysis of variance (ANOVA) and Bonferroni post-tests. *P*-value < 0.05 was considered as statistically significant.

3. Results

3.1. Synthesis of SKM13-2HCl

SKM13-2HCl was synthesized as following the scheme presented in Fig. 1. In comparison with our previous published antimalarial compound SKM13, the synthetic method was improved by using palladium-mediated coupling of amine (3) and 4,7-dichloroquinoline for the synthesis of Weinreb's amide (4). By developing a novel route for pure amide (4), the purity of SKM13 was greatly increased. SKM13-2HCl salt was readily obtained by the treatment of HCl in 1,4-dioxane.

3.2. In vitro cytotoxicity and inhibitory activity of SKM13-2HCl on Plasmodium falciparum

Cytotoxic concentration of the compound to cause death to 50% of viable cells in the host (CC_{50}) is an important factor to consider when developing any novel antipathogen compound. In here, we accessed the CC_{50} of novel compound in Vero cell (Table 1, Fig. S1A), which showed 48.81 ± 9.21 (Mean ± SD) μM for SKM13-2HCl, it is higher than our previous compound SKM13 (33.60 ± 7.07 μM), and control drug DHA (31.29 ± 8.94 μM). These data may indicate that the compound SKM13-2HCl in our current study is safer and have less cytotoxicity compare to SKM13 and DHA, but still more cytotoxic than CQ (120.00 ± 16.35 μM).

To explore the antimalarial activity of SKM13-2HCl against *Plasmodium falciparum* blood stage, we used the *Plasmodium falciparum* 3D7, a chloroquine susceptibility strain, to expose to drugs for 48hrs and parasitemia influenced by drugs were determined by FACS analysis. It showed that SKM13-2HCl has highest IC_{50} value and relative IC_{50} compare to CQ, DHA, and SKM13 in 3D7 (Fig. S1B). The IC_{50} with CQ sensitive parasite strain -*P. f* 3D7 was at 30.09 ± 1.06 nM, 29.59 ± 1.05 nM, 5.69 ± 0.35 nM, and 5.05 ± 0.15 nM for SKM13-2HCl, SKM13, DHA, and CQ, respectively (Table 1). Meanwhile, The IC_{50} of these drugs with CQ resistant parasite strain *P. f* K1 was at 141.8 ± 56.73 nM, 144.8 ± 18.56 nM for SKM13-2HCl and SKM13 drug; 33.98 ± 07.66 nM for DHA, and 66.20 ± 10.06 nM for CQ. The relative IC_{50} indicated that SKM13 and SKM13-2HCl showed 2.14 and 2.19 folds higher than CQ drug (Table 1).

In combination, the selective index (SI) value (CC_{50}/IC_{50}), which the higher value indicates the better performance of compound, even though, SKM13-2HCl has lower value than CQ, and DHA but it is better than original SKM13. The SI value was at 1622.05, 1135.35, 5499.35, and 23762.92 for SKM13-2HCl, SKM13, DHA, and CQ, respectively (Table 1). All raw data for IC_{50} measurement by FACS assay were shown in Fig. S2.

3.3. Inhibitory effect of antimalaria compounds on parasitemia of Plasmodium berghei NAT with single administration per day

Antimalarial activity of SKM13 was assessed in animal model in our previous study using rodent *Plasmodium berghei* NK65 and NAT strains (Yeo et al., 2017). To compare the relative difference of HCl salt of SKM13 to original SKM13 template compound, *P.beghei* NAT (atovaquone-resistant strain) was chosen in current study. The anti-malarial activity of SKM13 in a rodent model was assessed by a 4-day suppression test of 20 and 30 mg/kg of SKM13 in mice by once administration. In our previous study, we administrated SKM13 by twice per day (Yeo et al., 2017) but in this study, once per day was applied to test the efficacy of each drug.

Fig. 2 reveals the inhibitory effect of intravenous (i.v.) administration of SKM13 and SKM13-2HCl on *P. berghei* NAT growth *in vivo*. Daily increase in parasitemia was recorded and it was significantly increased in the infected-untreated group ($p < 0.001$) (Fig. 2 A - **B**) from 7 dpi. Meanwhile, i.v. treatments with chloroquine (CQ) at 10 mg/kg significantly reduced ($p < 0.001$) parasite density during all observation period. As *P. berghei* (NAT) belongs to atovaquone resistant strain, i.v. treatment of atovaquone at 4 mg/kg did not suppress parasitemia density.

It was interesting that SKM13 showed the increased parasitemia even at 30 mg/kg but 30 mg/kg SKM13-2HCl was effective to suppress parasitemia, indicating that suppressive effect of SKM13 on parasitemia was less effective than that of SKM13-2HCl. All of raw data for the observation of parasitemia for 16 days were presented in Fig. S3.

Body weight decreased in the parasite-infected mice without any treatment and atovaquone-treated group. At the lowest tested drug concentration (10 mg/kg) of SKM13 and SKM13-2HCl, mice showed significantly decreased body weight to 26.57 ± 1.43 g, (mean ± SD) in SKM13 treatment and 27.29 ± 1.64 g in SKM13-2HCl treatment at 2 dpi ($p < 0.001$) from 27.68 ± 1.28 g, 27.67 ± 1.97 g at 0 dpi, but the body weight of these group were higher than non-treatment and atovaquone treated group at 15 dpi, demonstrating that SKM13 and SKM13-2HCl at their lowest dose (10 mg/kg) were effective to tolerate parasite effect on mouse body weight and maintain the normal mouse metabolism until 15 dpi than non-treatment and atovaquone-treatment group.

CQ (10 mg/kg), and higher dose of both SKM13 and SKM13-2HCl (SKM13 (30 mg/kg), SKM13-2HCl (20 and 30 mg/kg)) treatment are all maintained normal mouse body weight.

3.4. Curative effect of antimalaria compounds on survival of Plasmodium berghei NAT-infected rodent with single administration per day

Analysis of survival rate showed that SKM13-2HCl was effective in treating *P. berghei* infection over SKM13 (Fig. 3). The parasite-infected group without treatment showed 33% survival rate at 15 dpi. CQ cured all mouse, showing 100% survival rate while Atovaquone had 33% survival rate. One administration of SKM13 (20 mg/kg) per day had 90% survival rate but SKM13-2HCl maintained 100% survival even at 20 mg/kg of single dose. It implies that SKM13-2HCl possessed the higher efficacy than SKM13.

3.5. Effect of the SKM13-2HCl treatment on hematological parameters

Changes in blood cell count are characteristic of *Plasmodium* infection and haematological changes during the course of a malaria infection, such as anaemia, thrombocytopaenia, leukocytosis, or leucopenia, are well recognized although these alterations vary with the level of malarial endemicity, background hemoglobinopathy, nutritional status, demographic factors, and also malaria immunity (Kotepui et al., 2015). *P. berghei* NAT strains significantly increased WBC counts ($p < 0.05$) at 9 dpi and progressively increased WBC counts were observed until 12 dpi ($p < 0.001$) and atovaquone treatment showed the same pattern of haematological result. In contrast, CQ and all SKM13 derivatives treatment did not affect WBC counts, HCT and Hgb (Fig. 4).

3.6. Effect of the SKM13-2HCl treatment on pharmacokinetics (PK)

Understanding the concept of half-life is useful for determining excretion rates as well as steady-state concentrations for any specific

Table 1
Summary of In vitro cytotoxicity and antimalarial activity of each compounds.

Strain	Compound	CC_{50}, μM	IC_{50}, nM	Relative IC_{50}	Selective index (SI) (CC_{50}/IC_{50})	Relative SI
			Ring stage			
3D7 (CQ Sensitive)	Chloroquine	120.00 ± 16.35	5.05 ± 0.15	1.00	23762.92	20.93
	DHA	31.29 ± 8.94	5.69 ± 0.35	1.13	5499.35	4.84
	SKM13	33.60 ± 7.07	29.59 ± 1.05	5.86	1135.35	1.00
	SKM13-2HCl	48.81 ± 9.21	30.09 ± 1.06	5.96	1622.05	1.43
K1 (CQ Resistant)	Chloroquine	120.00 ± 16.35	66.20 ± 10.06	1.00	1812.82	7.81
	DHA	31.29 ± 8.94	33.98 ± 07.66	0.51	920.91	3.97
	SKM13	33.60 ± 7.07	144.8 ± 18.56	2.19	231.98	1.00
	SKM13-2HCl	48.81 ± 9.21	141.8 ± 56.73	2.14	344.24	1.48

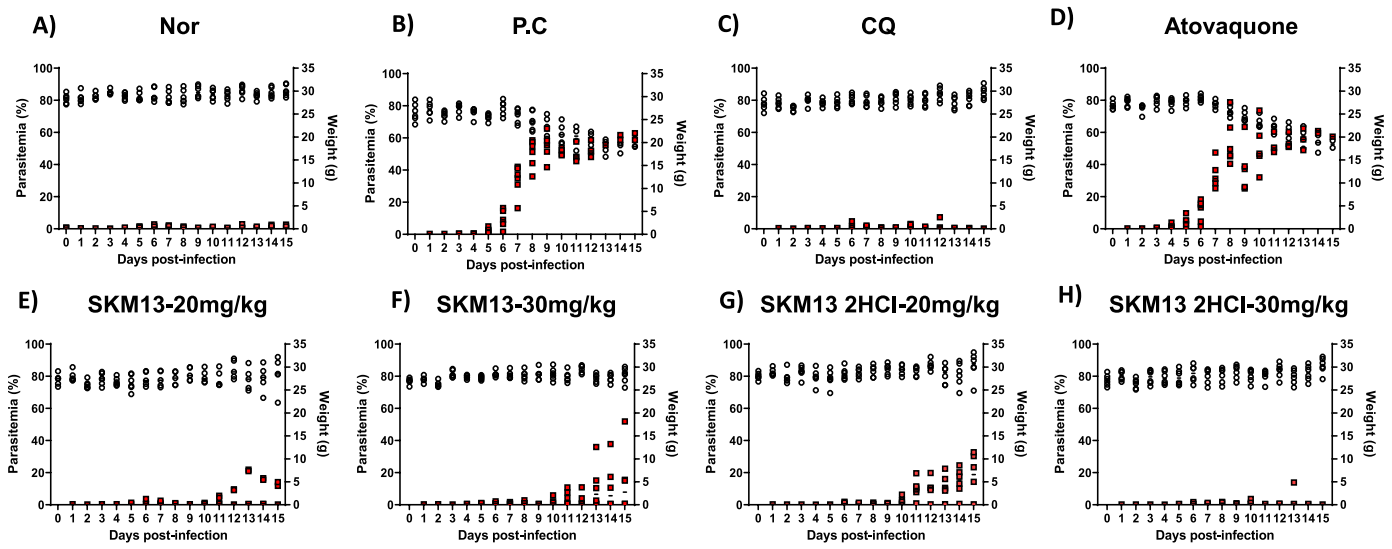

Fig. 2. Curative effect of antimalarial compounds against atovaquone-resistant strain *P. berghei* (NAT) in a 4-day suppression test. Parasitemia (red square) of *P. berghei* NAT and body weight (white circle) in *P. berghei* NAT-infected mice ($n = 6$) after treatment once per day. (**A**, Normal mice; **B**, *P. berghei* -infected group; **C**, chloroquine treatment; **D**, atovaquone treatment; **E** and **F**, SKM13 treatment at 20, 30 mg/mL (once per day); **G** and **H**, SKM13-2HCl treatment at 20 and 30 mg/kg (once per day). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

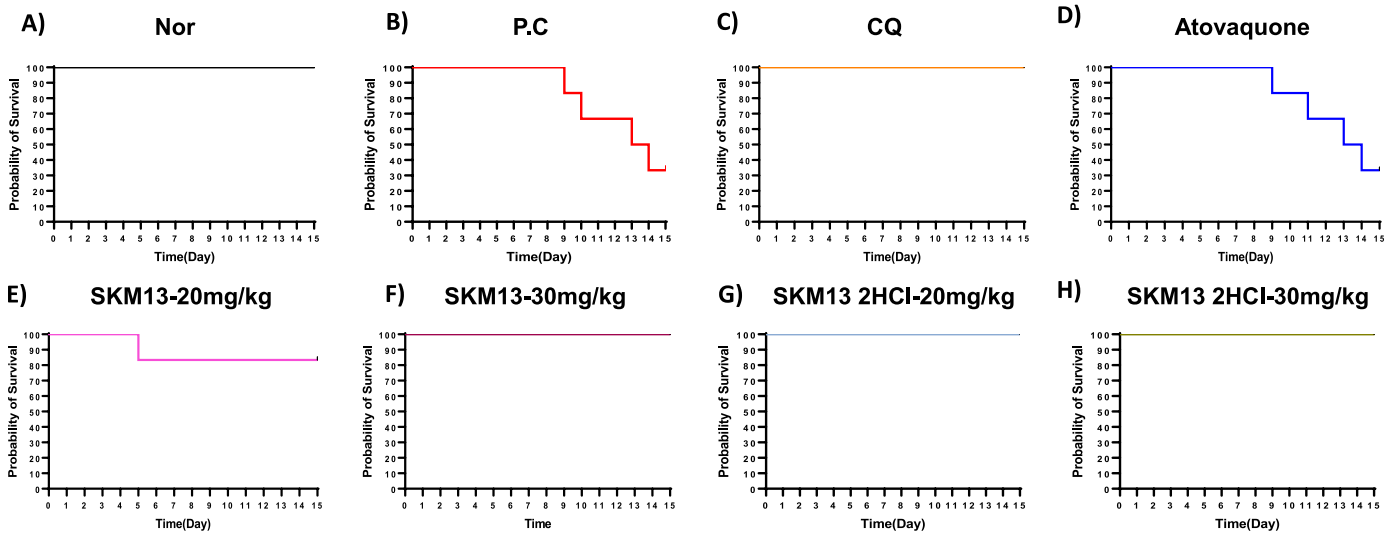

Fig. 3. Curative effect of antimalarial compounds against atovaquine-resistant strain *P. berghei* (NAT) in a 4-day suppression test. Survival rate of *P. berghei* NAT - infected mice ($n = 6$) was observed during 15 dpi after treatment once per day. (**A**, Normal mice; **B**, *P. berghei* -infected group; **C**, chloroquine (CQ) treatment; **D**, atovaquone treatment; **E** and **F**, SKM13 treatment at 20, 30 mg/mL (once per day); **G** and **H**, SKM13-2HCl treatment at 20 and 30 mg/kg (once per day).

drug (Erkent and Koytchev, 2008).

Half-life elimination is graphically represented with elimination curves that track the amount of a drug in the healthy mice body over time, typically with time on the independent axis and drug plasma concentration on the dependent axis, as shown in Fig. 5. C_{max} is the maximum serum concentration that a drug achieves in a specificied test area of the body after the drug has been administered. The highest C_{max} of SKM13 was shown in 1 h and the C_{max} was 63.79 ± 65.19 (mean ± SD) ng/mL. In contrast, the highest C_{max} of SKM13-2HCl was shown in 2 h and the C_{max} was 10.50 ± 5.97 (mean ± SD) ng/mL. SKM13 showed the variable C_{max} over SKM13-2HCl, indicating the inconsistent solubility of SKM13 than SKM13-2HCl in mice body.

Total drug exposure over time is represented in these graphs as the integral area under the curve (AUC). As summarized in Table 2, SKM13′AUC for the length of this study was 147.83 ng/mL * 60 min. In contrast, the AUC for SKM13-2HCl-treated infected mice was significantly lower at 30.14 ng/mL * 60 min. The solubility of SKM13 in water was 389 μg/mL, and SKM13-2HCl was 417 mg/mL by Ultra-High-Performance Liquid Chromatography (UHPLC). Raw data was shown in Tables S2–3.

3.7. SKM13 and SKM13-2HCl treatment increases an ER stress response

Multi-target drugs have raised considerable interest in the last decade owing to their advantages in the treatment of complex diseases and health conditions linked to drug resistance issues (Talevi, 2015). One of multi-target drugs is Artemisinin which is damaging protein and inhibiting the proteasome to inhibit malaria parasite via ER-stress response and CQ was reported not to involving ER stress (Bridgford et al., 2018). To determine if SKM13 derivatives was involving ER-stress unlike CQ, phosphorylation of eIF2α was assessed in the presence of those drugs and DHA was used as positive control of phosphorylation of

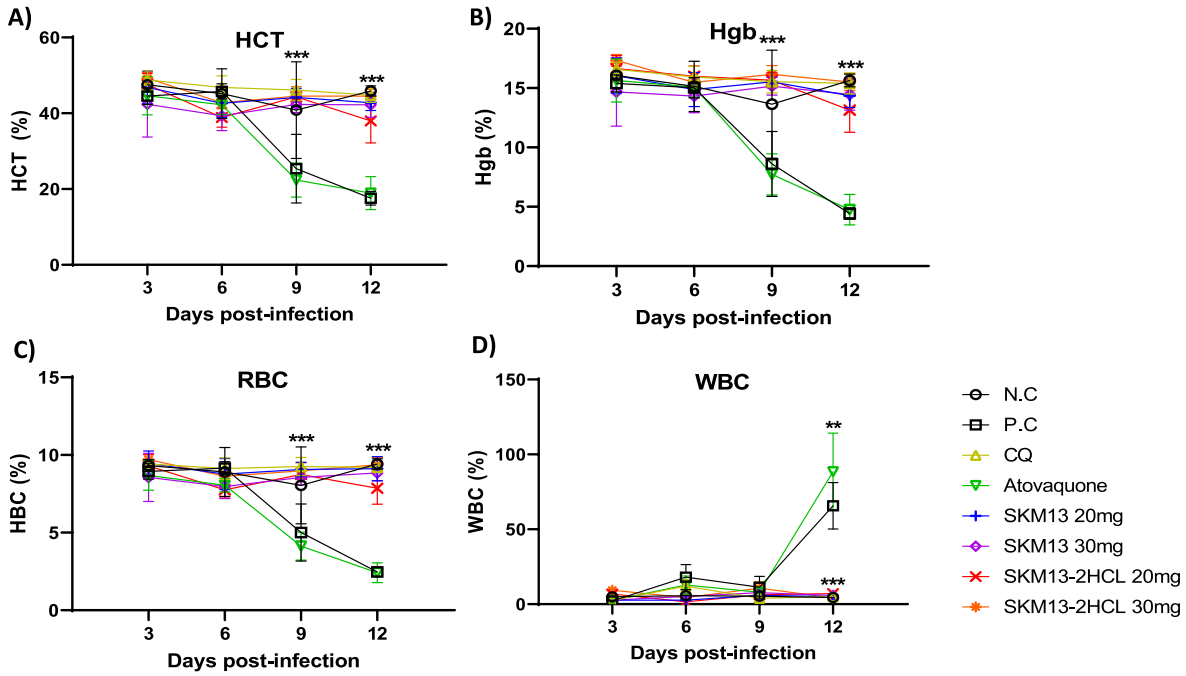

Fig. 4. Effect of SKM13 on haematological parameters of *P. berghei*. The effect of SKM13 on haematological indices was examined every 3 days after treatment (*$p < 0.05$, ***$p < 0.0001$). The values shown are the mean ± SD. (**A**) HCT, hematocrit; (**B**) Hgb, hemoglobin; (**C**) RBC, red blood cell; (**D**) WBC, white blood cell. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

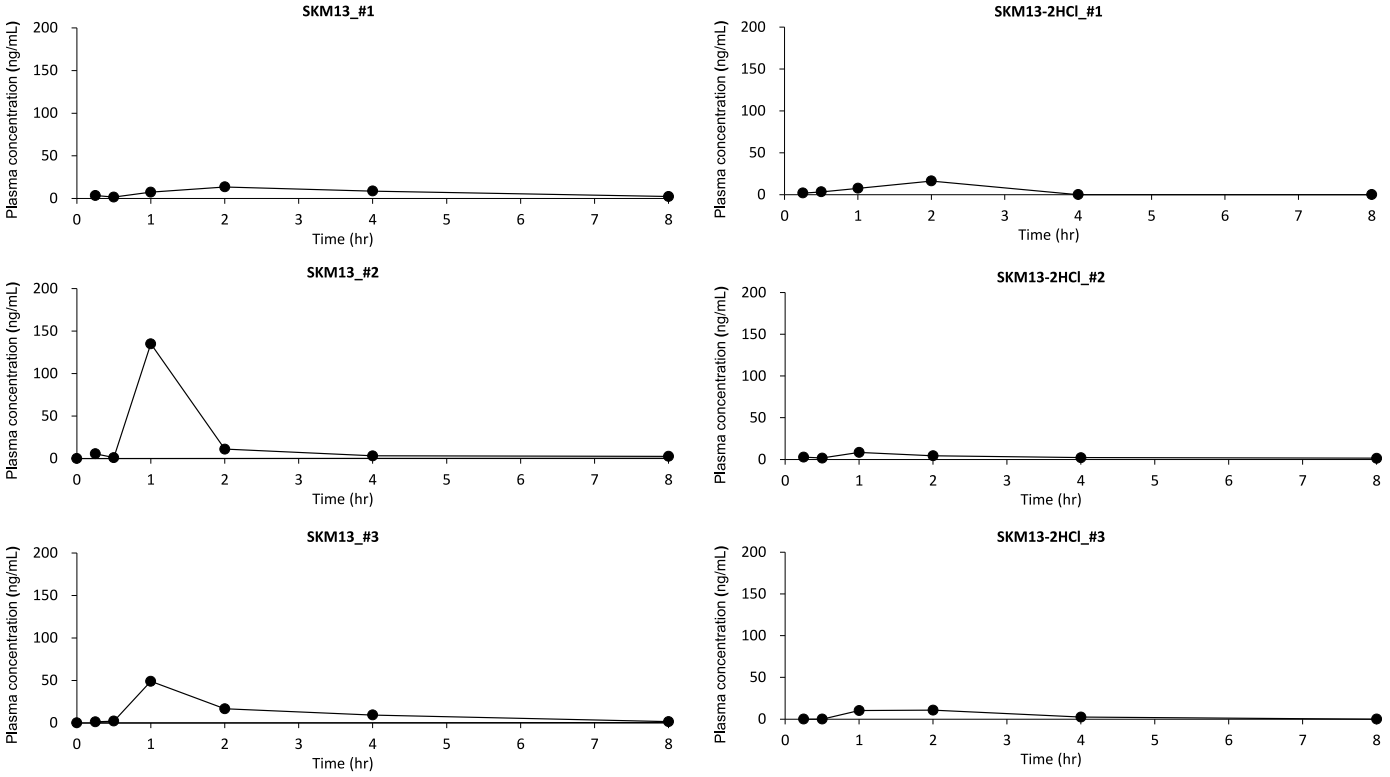

Fig. 5. Pharmacokinetics of SKM13 (left) and SKM13-2HCl (right) in plasma of healthy ICR mice. Mice ($n = 3$ per time point per treatment group) were orally gavaged with drugs to deliver 5 mg/kg body weight dose of drugs.

eIF2α. As seen in Fig. 6A, both of SKM13 and SKM13-2HCl increased phosphorylation of eIF2α. In contrast, CQ did not increase phosphorylation of eIF2α (Fig. 6B), implicating that SKM13 derivatives may possess the distinct mode of mechanism to kill parasite.

4. Discussion

Application of quinoline-based compounds for the treatment of malaria infections is hampered by drug resistance. Drug resistance has led to the combination of quinolines with other classes of antimalarials

Table 2
Pharmacokinetic parameters for SKM13 and SKM13-2HCl after oral gavage of healthy mice.

Parameter	SKM13	SKM13-2HCl
AUC_ng/mL * h	147.83436	30.13609
C_{max}_ng/mL	63.79200	10.49867
T_{max}_h	1.00000	2.00000
$t_{1/2}$_h	8.83062	1.45707

resulting in enhanced therapeutic outcomes (Nqoro et al., 2017).

Quinoline-novel target based hybrid molecules, is widely indicated, because the quinoline entity can inhibit β-haematin formation and contribute to a remarkable drug concentration within the digestive vacuole of *Plasmodium* (Pagola et al., 2000).

Previously, we found that quinoline template containing modified side chains such as α,β-unsaturated amides and phenylmethyl group were effective to control *Plasmodium spps.* in rodent model (Yeo et al., 2017).

Preferred choice of the salt form of a drug was reported to greatly affect the aqueous solubility and formulation of the compound as anti-malaria drug (Agharkar et al., 1976) but the characterization of being salt form on parasite growth as well as *in vivo* differential efficacy to increase the infected animal survival is rare.

There is one report to show that salt compounds were found to display good antimalarial efficacy *in vivo* against *Plasmodium berghei* (Takasu et al., 2007).

When SKM13 free amine was dissolved in DMSO and injected into the mouse, it seemed to cause uncomfortable behavior, such as mice not moving for a few seconds. This might be due to a large amount of DMSO, or pain from a mismatch between the pH of the mouse blood and the organic solvent. Another concern was the possibility that a significant amount of SKM13 free amine could be crystallized at the injection site due to the low water solubility. In this case, the distribution of SKM13 throughout the body takes a lot of time, and it may delay or make it difficult to reach the appropriate concentration as observed in *in vitro* experiment. To address and confirm these concerns, HCL salts of SKM13 were synthesized, and as expected, pharmacological activity in *in vivo* research much increased compared to SKM13. This is also a proof that SKM13's original efficacy was not properly measured in previous studies.

Inappropriate activation of *Plasmodium*/mammalian target of anti-malaria lead various cellular consequences including elevated endoplasmic reticulum (ER) stress (Shibeshi et al., 2020).

ER stress has been reported by artemisinin, the most potent anti-malaria drug which has multiple pathways being targeted but CQ did not induce ER-stress (Ismail et al., 2016; Bridgford et al., 2018). As SKM13 derivatives were designed with CQ template, different mode of action was anticipated in SKM13 compared to CQ.

Dihydroartemisinin (DHA), artemisinin and its derivatives, initiated ER stress response to kill parasites via a two-pronged mechanism, causing protein damage, and compromising parasite proteasome function. The consequent accumulation of proteasome substrates, i.e., unfolded/damaged and polyubiquitinated proteins, activates the ER stress response and underpins DHA-mediated killing (Bridgford et al., 2018).

Interestingly, our both SKM13 derivatives induced eIF2α phosphorylation like DHA, implicating that SKM13 may possess the distinct mode of action compared to CQ and thus, we propose that it may be efficient way to develop novel drugs against CQ-resistant strains.

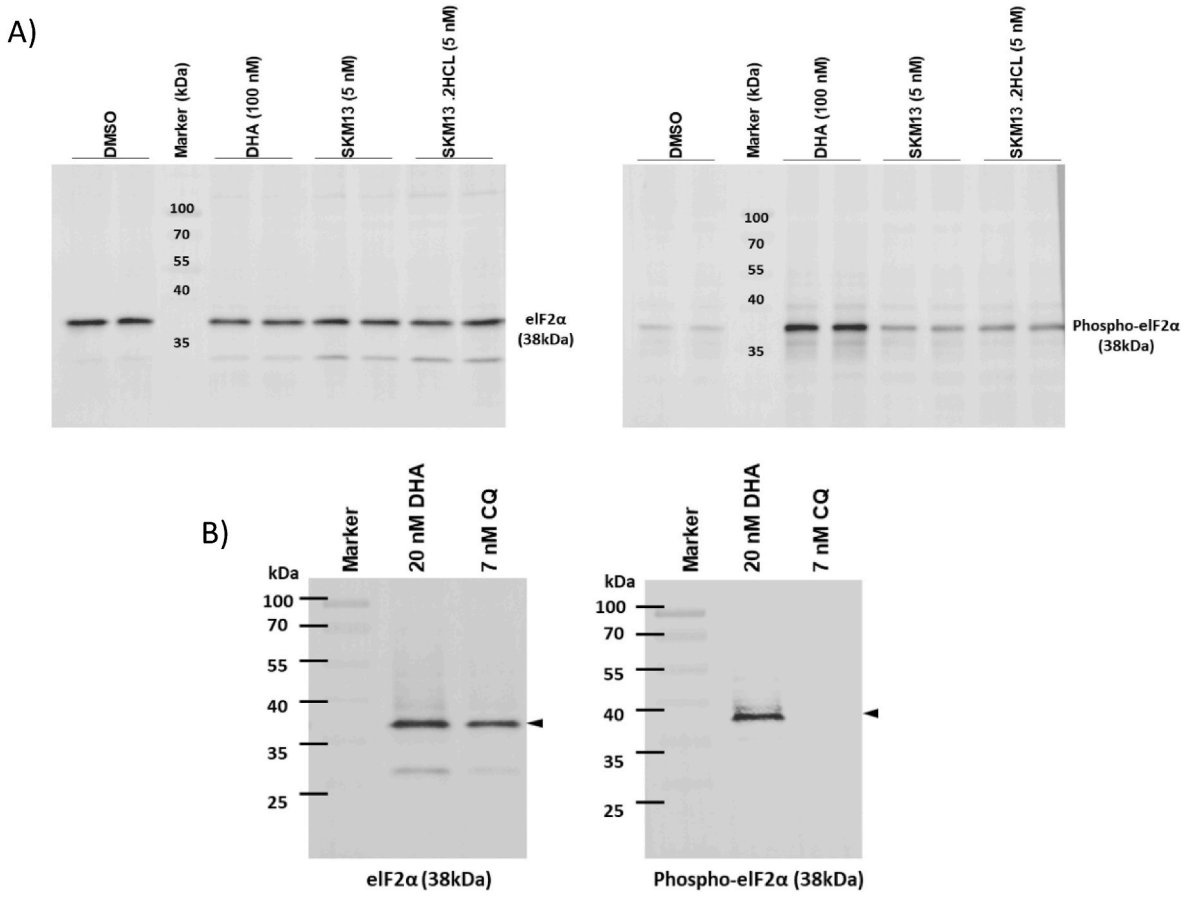

Fig. 6. Effect of SKM13 and SKM13-2HCl on phosphorylation of eIF2α. Trophozoites were treated with 0.00014% DMSO (mock), DHA or both drugs for 90 min and harvested immediately before lysates were subjected to Western blot analysis and membranes probed for phosphorylated-eIF2α.

Although the selective index (SI) value (CC_{50}/IC_{50}) between SKM13 and SKM13-2HCl has similar range but mouse study showed the better efficacy of SKM13-2HCl with single administration per day. It may be caused from stability of salt form of SKM13 *in vivo*.

The primary objective of treating severe malaria is to save life. Speed of response is important as this reflects the rate at which the disease is controlled, contributing to reduction in the risk to progress into the severe malaria. Therapeutic response in malaria is determined by the concentration profile (pharmacokinetics (Hopkins et al.)) of active antimalarial drug or drugs in the blood (White, 2013).

One of the major hurdles in developing therapeutic agents is the difficulty in delivering drugs through the intestinal mucosa and the intestinal mucosa barrier is formed by epithelial cells and is an impediment to oral drug absorption (Laksitorini et al., 2014). To improve drug delivery through these biological barriers, efforts have been made to alter the physicochemical properties of drugs as well as to alter the properties of the barriers via formulation design.

Therefore, understanding PK is a key to develop the improved antimalarial drug candidate. The lower C_{max} of SKM13-2HCl may be caused from gaining more hydrophilic characterization of SKM13. It implies that the hydrophobic SKM13 could penetrate intestinal mucosa barrier to arrive at vein but hydrophilic SKM13 (SKM13-2HCl) could have lower efficiency to penetrate it. In our study, PK measurement supported that SKM13 maintained the residual amount in blood over SKM13-2HCl, corresponding to the above theory. Balance of hydrophobic and hydrophilic characterization of drug could be the efficient direction to develop the improved antimalarial candidate following PK properties.

Our work identified the advantage of salt form of SKM13-2HCl over SKM13 *in vivo* study and it could be an orientation of development of novel antimalaria.

Author contributions

Thuy-Tien Thi Trinh, Performed research; Su-Yeon Yun, Performed research; Gum-Ju Bae, Performed research; Kwonmo Moon, Contributed new reagents or analytic tools; Hyelee Hong, Contributed new reagents or analytic tools; Tae Hui Eum, Contributed new reagents or analytic tools, Young-ah Kim, Contributed new reagents or analytic tools; Soon-Ai Kim, Contributed new reagents or analytic tools; Hyun Park, Analyzed data, Designed research; Hak Sung Kim, Designed research, Wrote the paper; Seon-Ju Yeo, Designed research, Wrote the paper.

Declaration of competing interest

There is no competing interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijpddr.2022.10.006.

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