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Transdermal delivery of artemisinins for treatment of pre-clinical cerebral malaria

Johanna Zech [a], Ron Dzikowski [b], Karina Simantov [b], Jacob Golenser [b,*,1], Karsten Mäder [a,1,*]

[a] Institute of Pharmacy, Martin Luther University Halle-Wittenberg, Kurt-Mothes-Str. 3, 06120, Halle (Saale), Germany
[b] Department of Microbiology and Molecular Genetics, The Kuvin Centre for the Study of Infectious and Tropical Diseases, The Hebrew University of Jerusalem, Ein Kerem, Jerusalem, 91120, Israel

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ABSTRACT

Transdermal drug delivery avoids complications related to oral or parenteral delivery - the need for sterility, contamination, gastrointestinal side effects, patient unconsciousness or nausea and compliance. For malaria treatment, we demonstrate successful novel transdermal delivery of artemisone (ART) and artesunate. The incorporation of ART into a microemulsion (ME) overcomes the limitations of the lipophilic drug and provides high transcutaneous bioavailability. ART delivery to the blood (above 500 ng/ml) was proved by examining the sera from treated mice, using a bioassay in cultured *Plasmodium falciparum*. Skin spraying of ART-ME eliminated *P. berghei* ANKA in an infected mouse model of cerebral malaria (CM) and prevented CM, even after a late treatment with a relatively small amount of ART (13.3 mg/kg). For comparison, the artesunate (the most used commercial artemisinin) formulation was prepared as ART. However, ART-ME was about three times more efficient than artesunate-ME. The solubility and stability of ART in the ME, taken together with the successful transdermal delivery leading to animal recovery, suggest this formulation as a potential candidate for transdermal treatment of malaria.

1. Introduction

Artemisinin and its derivatives are as of yet unmatched in their ability to rapidly and effectively clear blood-stage plasmodia in uncomplicated as well as severe human malaria, making them indispensable first-line antimalarial drugs (Phyo and Von Seidlein, 2017). Artemisinins probably possess more than a single mechanism of action. However, it is commonly accepted that radical damage of macromolecules in the parasitized erythrocytes due to the reactivity of the peroxide bridge is the most important factor for the biological activity (Patel et al., 2021). Unfortunately, artemisinin resistance has emerged already a decade ago (Talman et al., 2019). To prevent the emergence of parasite resistance, the WHO recommends the use of artemisinins in combination with a longer-acting second antimalarial drug (WHO Global Malaria Programme, 2014). Unfortunately, reduced sensitivity to the artemisinin combination therapies has been reported as well (Müller et al., 2019).

Artemisone (ART), a comparatively novel artemisinin derivative (Nagelschmitz et al., 2008), proved superior to the already marketed artemisinins in the treatment of malaria in mouse models because it is not metabolized to dihydroartemisinin (which has a shorter half-life) and it is not a subject to severe first pass metabolism. ART was successfully applied by intraperitoneal injections of the solubilized drug in DMSO (Waknine-Grinberg et al., 2010), slow release from solid polymer implants or pasty polymers (Golenser et al, 2017, 2020) and the orally administered vesicular Pheroid® system (Steyn et al., 2011). ART delivered orally in orange juice reduced parasitemia in monkeys infected by *Plasmodium falciparum* but did not cure them (Obaldia et al., 2009). Serum from healthy human subjects who consumed fast releasing tablets containing ART had a pronounced ex-vivo activity against *P. falciparum* (Nagelschmitz et al., 2008).

The following problems are commonly associated with the use of artemisinins: (1) limited chemical stability in solution, (2) poor aqueous solubility, (3) food dependent oral absorption and (4) short half-life. Recently, we could show that several of these problems could be solved by the formulation of self-emulsifying drug delivery systems (Zech et al., 2020), which combine high solubility, drug stability and simple preparation. ART administered in microemulsion (ART-ME) was

* Corresponding authors.
E-mail addresses: jacobg@ekmd.huji.ac.il (J. Golenser), karsten.maeder@pharmazie.uni-halle.de (K. Mäder).
[1] Jacob Golenser and Karsten Mäder are equally contributed to this work.

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Table 1
Composition of excipients in the formulations (given as % wt.) and process of preparation.

	Kolliphor® HS15	Propylene glycol	Polysorb® ID 46	Capmul® MCM	PBS
SMEDDS	50	10	20	20	–
ME	10	2	4	4	80

effective against *P. berghei* ANKA in a mouse model of cerebral malaria (CM) when delivered either orally, i.p. or via the intranasal route. Intraperitoneal injections were less effective than gavage and intranasal treatment (Zech et al., 2021). In a preceding study, the high efficacy of orally administered ART-ME against schistosomiasis was shown (Zech et al., 2020).

For comatose or vomiting malaria patients, oral treatment is no possibility. While intranasal ART was successful in mice, relatively fast clearance from the nasal mucosa and a comparatively small area of the human nasal cavity suggest that it will be mainly suitable for pre-referral treatment. Transcutaneous drug delivery, therefore, presents a valid alternative. Transdermal therapeutic systems, such as contraceptive or fentanyl patches, are known to achieve steady systemic drug release over multiple days (Janssen Inc. Canada, 2008; Janssen Pharmaceuticals Inc., 2019). Such systems would combine several advantages in malaria therapy. Minimal parasiticidal drug serum concentrations maintained over more extended periods of time would not only increase drug effectiveness but also reduce the risk of acquiring parasite resistance (Kay et al., 2015). A non-invasive, easy application could allow the use even under non-sanitary conditions and - together with greatly reduced treatment frequency - improve compliance.

Various methods have been tried for the transdermal application of antimalarial drugs. Rodent malaria real prophylaxis was achieved by transdermal delivery of primaquine in a mixture of ethylcellulose and triglycerides (Mayorga et al., 1998). Klayman et al. developed a gel containing artelinic acid that was applied topically, twice a day, during three days post infection (total dose 270 mg/kg) and prevented mouse death of *P. berghei* infections (Klayman et al., 1991). In view of the amount needed for the effect and its alleged toxicity and because it is not preferable to the currently available artemisinins, artelinic acid did not enter clinical use (Li et al., 2007). However, an improved formulation containing artemether instead of artelinic acid examined in the same mouse model was efficient at a lower dose of a total of 60 mg/kg (Lin et al., 1994). Nnamini et al. developed a nanostructured lipid carrier for the topical application of artemether (Nnamani et al., 2014). The drug was released ex vivo through human epidermis over 46 h at a concentration of about 50 ng/ml/hour. Other groups used cataplasms containing artesunate and febrifugine (75 and 0.5 mg/kg, respectively) for the transcutaneous treatment of mice infected by *P. berghei* ANKA (Shen et al., 2015). Successful treatment during four days (starting immediately after infection) eliminated all parasites. Treatment of *P. berghei* infection in rats, using transdermally delivered oleanolic acid (OA) from an amidated pectin hydrogel patch that contained 34 mg OA/kg, completely reduced detectable parasitemia. Noteworthy is the fact that the treatment started late, seven days post infection, when the parasitemias were extremely high, at about 40 percent (Sibiya et al., 2016). A novel sublingual spray formulation of the antimalarial drug artemether was superior in pharmacokinetics to the drug in tablets (Salman et al., 2015).

Given the inflammatory nature of cerebral malaria (CM), immune modulation (Cabrales et al., 2010; Waknine-Grinberg et al., 2013) was proposed for preventing experimental CM. Cerebrovascular constriction is associated with local inflammation in CM. Therefore, the transdermal delivery of glyceryl trinitrate is a rational approach to induce vasodilution. This treatment was effective at preventing CM and extending mice survival times (Orjuela-Sánchez et al., 2013).

For the transdermal application of ART, classical oil in water nanoemulsions were developed and examined in vitro. ART reached the stratum corneum of human skin but was not successfully delivered transdermally (Burger et al., 2018). Similar results were found using ART encapsulated in solid lipid nanoparticles that also reached the stratum corneum of human skin (Dwivedi et al., 2016). These last two methods might be more useful for the treatment of skin lesions or melanoma/skin cancer (Wong et al., 2020). Other surfactant-based nanodispersions were unable to deliver ART into the skin (van Zyl et al., 2019).

The aim of the current study is to investigate the efficacy and demonstrate the feasibility of transcutaneous delivery of artemisone for the treatment of CM in a rodent model, by using our recently developed microemulsion delivery system that is made of safe, non-toxic excipients (Zech et al., 2020, 2021). Because of the clinical importance of artesunate for the treatment of severe malaria, we also investigated artesunate loaded ME to compare the suitability of transdermal delivery for both artemisone and artesunate.

2. Materials and methods

2.1. Materials

Artemisone (ART) was donated by Cipla, Mumbai, India. Artesunate was bought from Sigma, Israel.

Polysorb® ID 46 (Isosorbide caprylocaprate diester) was a gift from Roquette, Lestrem, France.

Capmul® MCM EP (Glycerol monocaprylocaprate) was supplied by Abitec, Columbus, USA.

Kolliphor® HS15 (Polyoxyl (15) Hydroxystearate) was obtained from BASF, Ludwigshafen, Germany and molten before use. Propylene glycol was manufactured by Caesar & Lorentz GmbH, Hilden, Germany.

Phosphate buffered saline (PBS) pH 7.4 was bought from Biological Industries, Bet Haemek, Israel.

All other chemicals were of analytical grade or higher, and only double distilled water was used in the experiments.

2.1.1. Formulation of the drug delivery system

We developed a simple self-microemulsifying drug delivery system (SMEDDS) for ART based on a combination of polar lipid excipients (Table 1). The composition of the SMEDDS provides high solubility (59 mg/g) and stability of ART. No drug degradation was observed even 3 months after a storage at 30 °C (Zech et al., 2020). The transparent and water free SMEDDS formulation is formed by simple mixing of the excipients. Drug containing SMEDDS are obtained by dissolving of the drug in the SMEDDS formulation. A detailed description of the formulation development and its physicochemical characterization can be found in Zech et al. (2020).

For the spray treatment in this work, we used a SMEDDS-derived microemulsion (ME) with a water content of 80%. The ME

Table 2
The effect of serum collected from mice treated by ART-ME on cultured *P. falciparum*.

Percent reduction of parasitaemia			
Serum dilution	1/900	1/300	1/100
Mouse serum control	9.8 (t + s)	26.0 (t + s)	66.2 (r + dt)
One hour post treatment by ART-ME	100 (np)	100 (np)	100 (np)
Three hours post treatment by ART-ME	84.9 (r)	90.9 (dr)	97.1 (dr)
Five hours post treatment by ART-ME	53.2 (r + t + s)	83.2 (r + dt)	93.7 (dr)
Free ART	1 ng/ml	2 ng/ml	4 ng/ml
	44.6 (r + t)	86.4 (r + dr)	100 (np)

n = 3/group. Results are expressed as percent reduction of parasitemia compared to untreated control (without serum). Control parasitemia in untreated cultures was 1.85%
np = no parasites; r = ring; dr = dead (looking) ring; t = trophozoites; dt = dead (looking) trophozoites; s = schizonts.
All results of serum collected from treated mice at all time points were significant compared to the effects of normal sera ($p < 0.05$); the effect of normal serum compared to cultures that did not contain serum was significant only at a serum concentration of 1/100.

formulation is produced by dilution of the drug containing SMEDDS with PBS. Immediately before administration, 4 ml PBS were added to each 1 g of ART-SMEDDS to obtain the ART-ME, which was then used for spraying/treatment of the animals (Table 1).

A spray volume of 150 µl was delivered per application on mice with an average weight of 25 g. The drug content in the applied volume of 150 µl was 0.11 mg, 0.33 mg and 1 mg. These amounts correspond to individual doses of 4.4/13.3/40 mg ART/kg body weight. These formulations are consequently referred to as ART-ME 4.4, ART-ME 13.3 and ART-ME 40. Artesunate was prepared identically and referred to accordingly. Drug-free formulation (placebo-ME) was used as placebo.

For *P. falciparum* in vitro cultures, the ED_{50} of ART-ME 40 was found to be 1–2 ng ART/ml (Zech et al., 2020).

2.1.2. Glass nasal-spray bottles

Glass bottles (Wepa Apothekenbedarf, Hillscheid, Germany) equipped with a nasal-spray pump that delivers 150 µl ME per spray were used in the treatment of the animals. Simple testing of spray plumes was performed for three pump heads by spraying dyed ME onto white paper from a fixed distance of 2.5 cm. Measuring the dyed area was done with the IC Measure imaging software (The Imaging Source Europe GmbH, Bremen, Germany). At this range, the sprayed area had a size of (3.4 ±

Table 3
The effect of placebo-ME vs ART-ME 40 spraying on the course of the disease.

Treatment	Days post infection	Treatments per day	Number mice with CM	Delayed death	Number mice survived
Untreated	–	–	27	1/28[a]	–
Placebo-ME	3–6	1	6	–	–
	3–6	2	4	8/12[b]	–
	4–6	2	6	–	–
ART-ME 40	3–6	1	–	–	5[c]
	4–6	1	–	–	6[c]
	3–5	2	–	–	6[c]
	5–8	2	–	–	5[c]

[a] Cumulative number of untreated mice.

[b] $p<0.05$ compared to untreated mice.

[c] $p < 0.001$ compared to untreated mice.

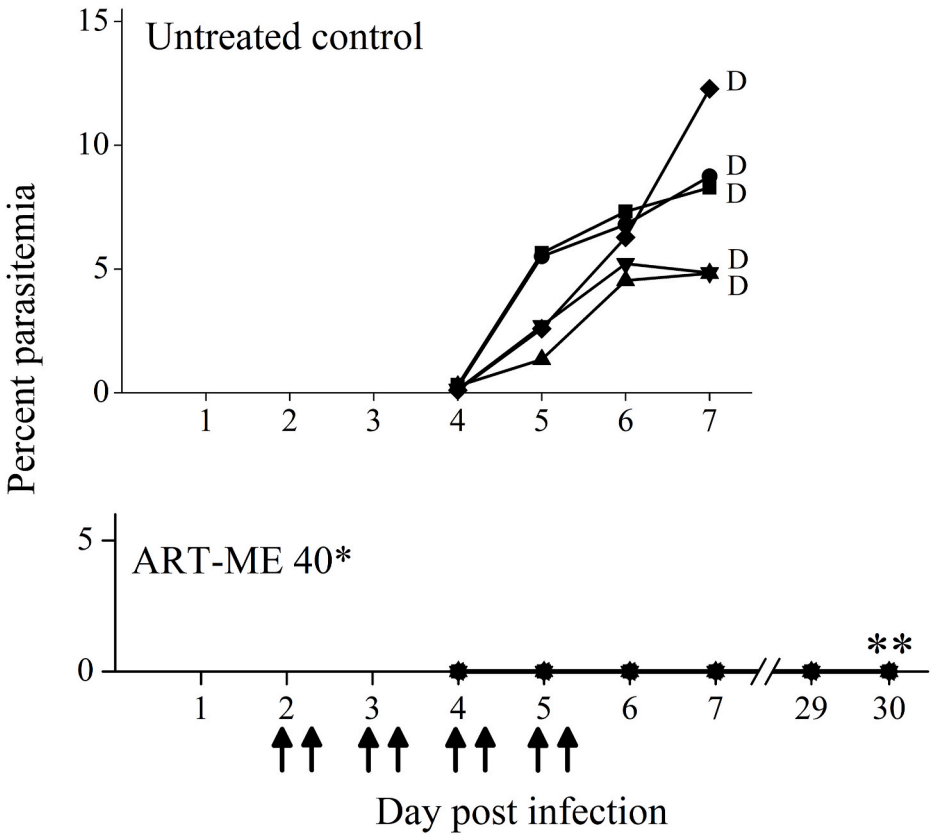

Fig. 1. The effect of early treatment with ART-ME (40 mg/kg body weight) on the course of the disease. A volume of 150 µl with an ART concentration of 1 mg/ml was sprayed twice daily, days 2-5 post infection. ↑ Spraying, the interval between treatments per day was 8 h; D = death; Each line represents one mouse, n = 6 for ME-ART, n = 5 for untreated control; $p < 0.01$ starting on day 5 post infection (parasitemias) and delay of death in all treated mice; * mg ART/kg bodyweight; ** Blood smears were negative throughout the experimental period of 30 days.

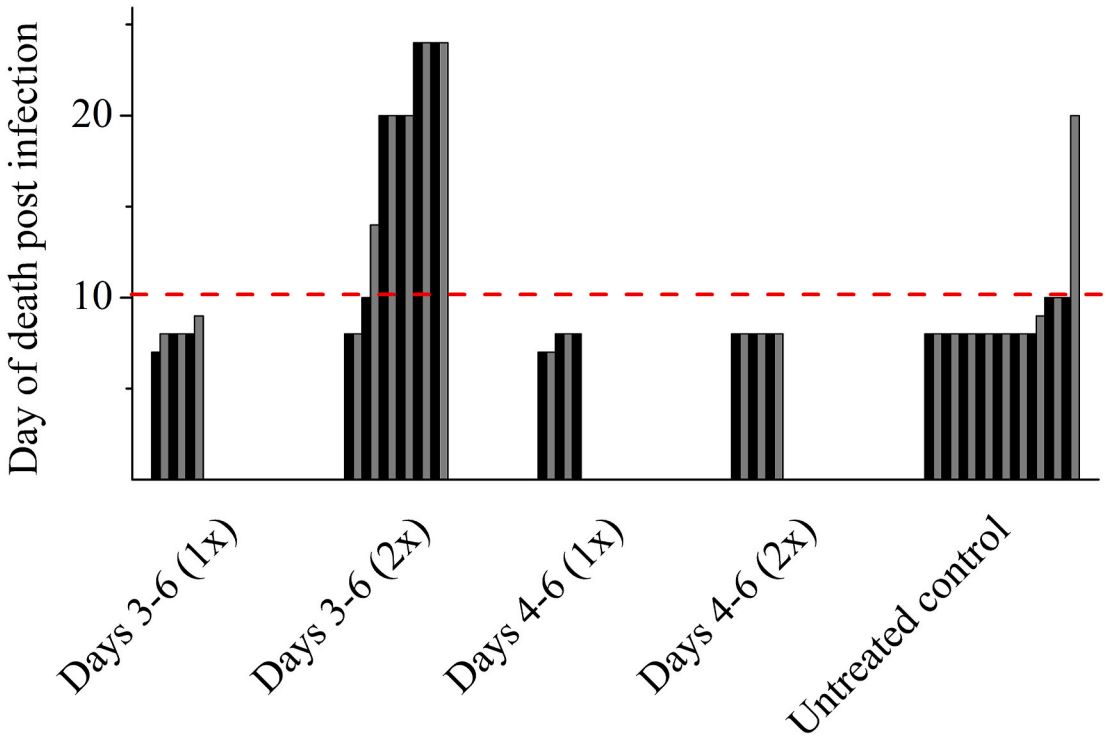

Fig. 2. The effect of placebo-ME on the course of the disease in treated vs untreated infected mice. The only significant effect (results shown above the dashed line) was obvious in mice treated twice by placebo-ME at 3–6 days post infection.

0.4) cm^2.

2.2. Animals

C57Bl/6 Ola-Hsd male mice (C57Bl/6, from Harlan, Israel), aged 7–8 weeks, were used in all experiments. The animals had unlimited access to food and water. They were housed in groups under standard conditions, and a 12 h on/off light cycle was maintained.

Experiments were performed in accordance with institutional guidelines for animal care, by protocols approved by the Animal Ethical Care Committee of the Hebrew University of Jerusalem (Protocol No. MD-12-1351; Golenser's accreditation No. 12180).

2.3. Methods

2.3.1. Culturing Plasmodium falciparum

P. falciparum 3D7 parasites were cultivated at 4% hematocrit in RPMI 1640 medium (Beit Haemek, Israel), 0.5% Albumax II (Invitrogen, Carlsbad, California, USA), 0.25% sodium bicarbonate, and 0.1 mg/ml gentamicin. Parasites were incubated at 37 °C in an atmosphere of 5% oxygen, 5% carbon dioxide and 90% nitrogen. Parasite levels were monitored by direct microscopic observation of Giemsa stained blood smears.

2.3.2. Bioassay

Artemisone was quantified in a sensitive bioassay based on a two-day culture of *P. falciparum*. The bioassay permits the detection of ART concentrations down to 1 ng/ml and can indicate which plasmodial stages are affected. Samples of blood were collected from mice in MiniCollect tubes (Greiner Bio-One, Germany) one, three and 5 h after treatment of naïve mice by spraying with two doses of ART-ME 40 (equals 300 µl, 80 mg ART/kg bodyweight). The serum was separated by centrifugation (5 min, 6000 RPM), sterilized by filtration, diluted and added to *P. falciparum* cultures in 96 flat bottom disposable sterile microplates (Costar, USA) that were incubated as detailed above (2.3.1).

2.3.3. Infection of animals

Mice were infected with *Plasmodium berghei* ANKA (MRA-311, CDC Atlanta) to cause experimental cerebral malaria.

The parasite strain was maintained by serial transfer of parasitized erythrocytes (PE) from infected donor mice to naïve mice. Intraperitoneal injection of experimental mice with 5×10^4 PE from peripheral blood of infected passage mice caused fatal cerebral malaria on day 6–10 post-infection (PI) in at least 90% of the infected animals.

2.3.4. Monitoring of infection

Parasitaemia in the infected mice was followed via light microscopy of Giemsa stained tail blood smears. The number of PE per 10,000 red blood cells was recorded.

2.3.5. Severe malaria mouse model

Male C57Bl/6 mice infected with *P. berghei* ANKA were used as a well-established model of experimental severe malaria (Oca et al., 2013). Besides a staring coat, hunching and apathy, untreated infected mice exhibited characteristic clinical signs indicative of CM, such as convulsions and coma.

Histology of hematoxylin and eosin-stained brain tissue revealed only a small number of parasites in the brain of the infected mice (data not shown). But intravenous injections of Evans Blue (0.2 ml of 1% in PBS/mouse) clearly indicated impairment of the blood-brain barrier by the infection (Golenser et al., 2020). While no staining was detectable in the brain tissue of naïve mice, the dye distinctly appeared in the brains of infected animals. Parasitemias of dying mice were below 20%. These animals were therefore categorized as suffering from CM and were sacrificed according to the instructions of the ethical committee (when they were about to die) at 6–9 days post infection. Mice were classed as "survived" if, after treatment, there was no reappearance of parasites until the end of the experiment, at least 28 days post infection.

2.3.6. Drug application by spraying

Mice were shaved in the mid-area of their backs (about 2.5 cm

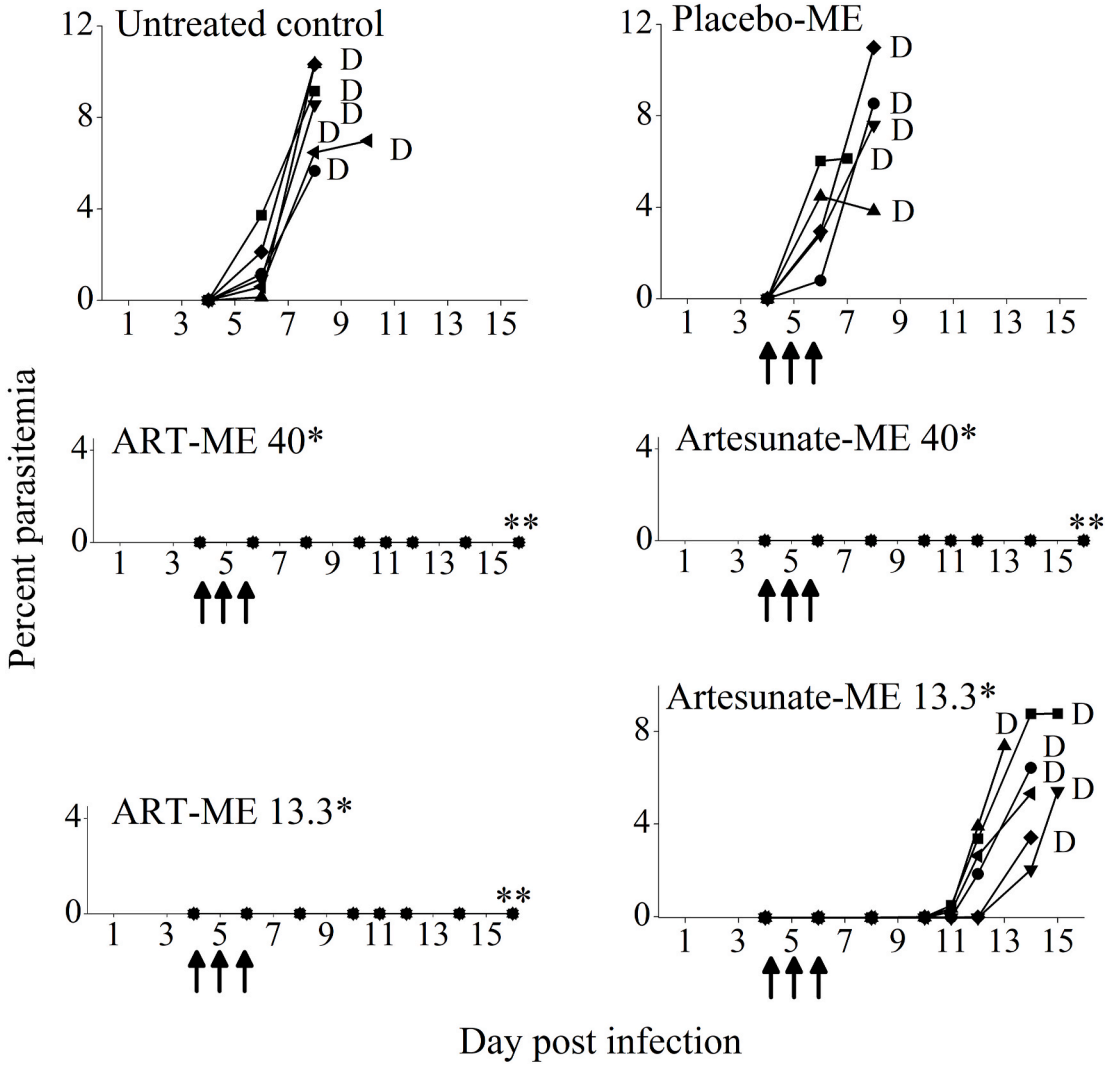

Fig. 3. Impact of artemisone vs. artesunate in a mouse model of cerebral malaria. ART-ME vs. artesunate-ME, effects on the course of the disease. ↑ ME spraying, arrow symbols; D = death; Each line represents one mouse; n = 6 in all groups except for placebo-ME where n = 5; * mg drug/kg bodyweight; ** Blood smears were negative throughout the experimental period of 30 days.

diameter circle) one or two days before drug application. The animals were treated by spraying placebo-ME, ART-ME or artesunate-ME on the shaved area using a nasal-spray bottle with an applicator that delivers 150 µl of the formulation for each spray. Mice were held on their backs so the spray bottle could be kept upright to guarantee the delivery of a consistent amount. The pump was primed before use, and the distance between the nozzle and animal skin was maintained at around 2.5 cm. Mice were not observed licking the sprayed area; no cleaning of the fur was indicated by the continuous greasy appearance of the application site. However, ingestion of some of the formulation cannot be completely ruled out.

Mice were treated either once or twice a day with 150 µl spray. The duration, dose and dosing frequency are indicated in the corresponding figure legends and tables.

3. Results

3.1. Serum concentration by bioassay

A preliminary experiment was conducted to examine whether drug delivery to the blood is feasible after topical spraying of ART-ME. A bioassay in cultured *P. falciparum* enabled the examination of ART availability in blood collected from treated mice. The mice were treated by two successive doses of ART-ME (equals 80 mg/kg ART) before blood sampling. One and 3 h post treatment, the collected serum totally eliminated *P. falciparum* parasitemia at 1/100 dilution and almost totally at 1/300. Serum collected after 5 h at this concentration reduced about 50% of the parasitemia, similar to the effect of 1 ng/ml free ART (Table 2). At this concentration, normal serum had no effect. This means that 5 h post infection the serum had an equivalent ART content of about 900 ng/ml. The results prove transdermal passage of active ART to the blood. Note that the inhibition of plasmodial development in culture was expressed by reduction of parasitemia and also by inhibition of intracellular development. For example, while untreated control cultures developed to a stage of advanced trophozoites and mature schizonts, serum collected 3 h post treatment at 1/900 concentration, killed most parasites and arrested the residual ones at a ring form stage.

3.2. Transdermal treatment with ART-ME

Following the demonstration of the feasibility of the transcutaneous treatment, an experiment was performed in which a group of infected mice was treated by spraying 150 µl ART-ME 40 twice a day, at day 2–5 post infection. Consequently, while all untreated mice died of CM seven days post infection, parasites could not be detected in the treated mice throughout the entire detection period, up to one month (Fig. 1).

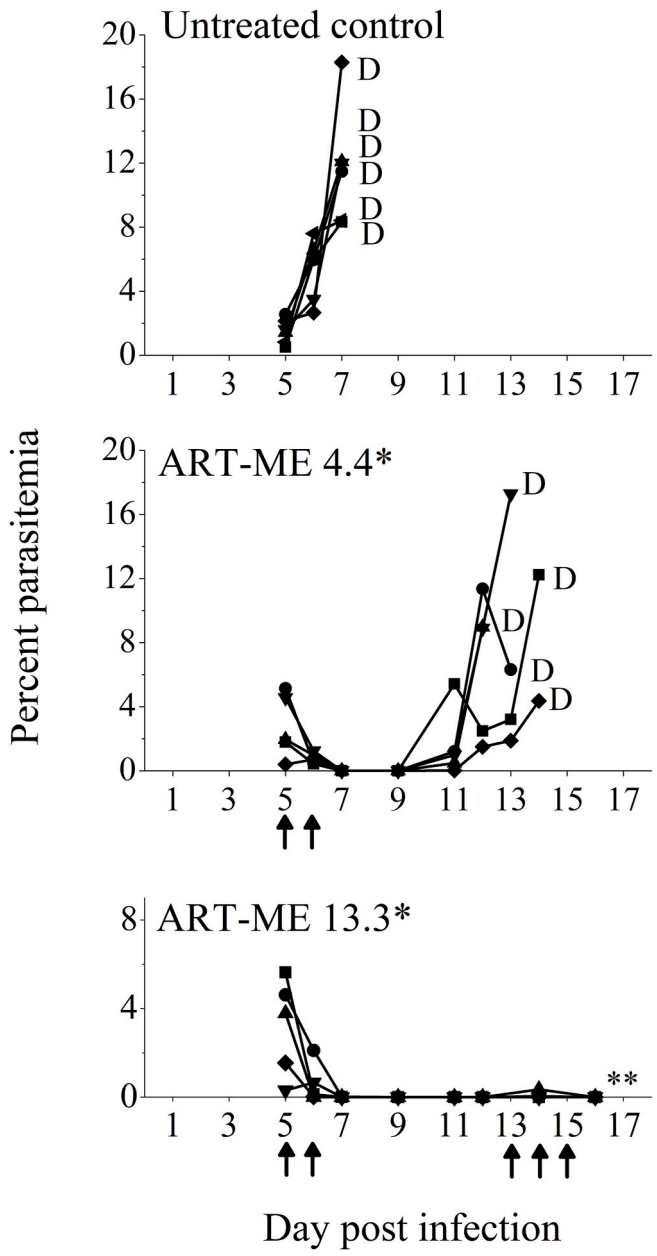

Fig. 4. Dose response of late treatment of ART-ME. ↑ ART-ME spraying; D = death; Each line represents one mouse; n = 5 in all groups except for the untreated mice where n = 6; * mg ART/kg bodyweight; ** Blood smears were negative up to the end of the experiment 28 days post infection.

3.3. Effect of placebo-ME

An experiment was conducted to evaluate the effect of placebo-ME alone vs ART-ME 40. Mice were treated once or twice a day, at different days post infection, with 150 µl placebo-ME or ART-ME 40. The results (Table 3) indicate that if treatment started two days post infection and was performed twice a day, the placebo-ME could delay the death. A single or delayed placebo-ME treatment starting four days post infection did not affect the course of the disease, and the mice died of CM, similar to the infected untreated mice (Fig. 2). ART-ME 40 at all combinations eliminated the parasites, and consequently, all treated mice survived even if treatment started five days post infection when symptoms of CM were already apparent.

3.4. ART-ME vs Artesunate-ME

The following experiment demonstrates a difference between the activity of ART and artesunate, both in ME. The drugs were applied only once on days 4–6 post infection to minimize ME effect.

The untreated control and the placebo microemulsion were similar (Fig. 3a top). Both artemisone and artesunate microemulsions showed bioactivity: at a dose of 40 mg/kg a complete elimination of the parasites was observed (Fig. 3, middle panels). The artemisone microemulsion killed all parasites even at a drug dose of 13.3 mg/kg (Fig. 3, bottom left panel). Artesunate showed less activity at this concentration however, a significant delay of death by about a week was observed (Fig. 3 bottom right panel).

Parasitemias and survival were significant for ART-ME in both concentrations compared to untreated and placebo-treated mice and for ART-ME 13.3 vs artesunate-ME 13.3 ($p < 0.001$). Artesunate-ME 40 was equally significant. Artesunate-ME 13.3 was less effective than artesunate-ME 40, still significant in delaying death compared to the controls ($p < 0.001$) but not significant in preventing death.

3.5. Reduced dose treatment

Given the results thus far, an additional experiment was performed to evaluate the possibility of reducing the ART dose and the number of treatments in a late stage of the disease. Mice were initially treated twice per day with ART-ME 4.4 and ART-ME 13.3 at days five and six post infection when CM symptoms were obvious. ART-ME 4.4 delayed mortality of CM by about a week. ART-ME 13.3 delayed the appearance of parasites until 14 days PI in 3/5 mice, and 2/5 mice were still negative at that time. We further treated all mice in this group with ART-ME 13.3 at days 13–15, and consequently, the mice cleared the parasites and stayed negative until the end of the experiment, two weeks later (Fig. 4).

4. Discussion

Transdermal drug delivery avoids complications related to parenteral injections (contamination, technical difficulty related to repeated intravenous injections) as well as oral treatment (gastrointestinal side effects, first pass metabolism, coma and vomiting) and compliance (Shen et al., 2015). Topical or transcutaneous treatment by spraying artemisinins in various formulations has been suggested for elevating allergy symptoms, anesthetic purposes, wounds healing, and skin cancer (Härtel et al., 2018; Li et al., 2012; Nnamani et al., 2014; Wong et al., 2020). Other transdermal methods of applying artemisinins (and other drugs) were suggested to treat malaria, mostly with limited success (see Introduction).

We present a new formulation that, by skin spraying, achieves successful transdermal delivery of the more recent drug artemisone (ART) and consequently prevents the development of experimental cerebral malaria. Compared to the oral treatment we described in (Zech et al., 2021), transdermal delivery is even more effective. There, gavage of the same dose of ART in the ME could only eradicate parasites in 30% of the mice, while transdermal treatment led to the recovery of all animals. Even a late treatment, starting five days post infection with a relatively small amount of ART (13.3 mg/kg), eliminated the plasmodia and prevented CM. Surprisingly, treatment with placebo delayed death in the infected mice but only if given twice a day, starting early and administered on days 3–6 post infection. As of now, we do not have an explanation for this observation, considering that the ME had no effect when given orally or intranasally or when tested against *P falciparum* in vitro (Zech et al, 2020, 2021).

Artesunate is the most commonly used commercial artemisinin against severe malaria. It is currently applied by injection. We formulated artesunate in our microemulsion system and observed pronounced antimalarial activity after transdermal application. However, the advantage of ART over artesunate, which had previously been shown by injections of drugs solubilized in DMSO (Waknine-Grinberg et al., 2010), was also consistent after transdermal application: ART was about three

times more efficient than artesunate. Nevertheless, the transdermal administration of the clinically used drug artesunate is also a promising option for the treatment of severe malaria.

The solubility and stability of ART in the ME (Zech et al., 2020), taken together with the successful transdermal delivery leading to animal recovery, suggest transcutaneous treatment with artemisinins as a potential alternative application route to i.v. and i.m. injections for the treatment for severe malaria.

In conclusion, effective transdermal delivery of artemisone and artesunate has been demonstrated in a preclinical mouse model of cerebral (severe) malaria. The developed formulation has a high potential for clinical translation because of its performance, which combines high drug solubility, stability and efficacy.

Declaration of competing interest

The authors have nothing to declare. There are no conflicts of interests.

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