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BLOOD COMPONENTS

TRANSFUSION

# Robust inactivation of *Plasmodium falciparum* in red blood cell concentrates using amustaline and glutathione pathogen reduction

**Cissé Sow**[1] | **Amélie Bouissou**[1] | **Yvette A. Girard**[2] | **Gurvani B. Singh**[2] | **Lotfi Bounaadja**[1] | **Jean-Marc Payrat**[3] | **Delphine Haas**[4] | **Hervé Isola**[4] | **Marion C. Lanteri**[2] | **Peter Bringmann**[2] | **Philippe Grellier**[1]

[1]UMR7245 MCAM, Muséum National d'Histoire Naturelle, Team PPL, CNRS, Paris, France

[2]Cerus Corporation, Concord, California, USA

[3]Cerus Europe BV, Amersfoort, Netherlands

[4]EFS Grand Est, Strasbourg, France

**Correspondence**
Philippe Grellier, MNHN, UMR 7245 MCAM, CP 52, 57 rue Cuvier, 75005, Paris, France.
Email: philippe.grellier@mnhn.fr

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## Abstract

**Background:** *Plasmodium falciparum* is the parasite responsible for most malaria cases globally. The risk of transfusion-transmitted malaria (TTM) is mitigated by donor deferrals and blood screening strategies, which adversely impact blood availability. Previous studies showed robust inactivation of *P. falciparum* using nucleic acid-targeting pathogen reduction technologies (PRT) for the treatment of plasma and platelet components or whole blood (WB). The efficacy of the amustaline–glutathione (GSH) PRT to inactivate *P. falciparum* is here evaluated in red blood cells (RBC), as well the impact of PRT on parasite loads, stages, and strains.

**Study Design and Methods:** RBC units resuspended in AS-1 or AS-5 additive solutions were spiked with ring stage-infected RBC and treated with the amustaline–GSH PRT. Parasite loads and viability were measured in samples at the time of contamination, and after treatment, using serial 10-fold dilutions of the samples in RBC cultures maintained for up to 4 weeks.

**Results:** *P. falciparum* viability assays allow for the detection of very low levels of parasite. Initial parasite titer was >5.2 $\log_{10}$/ml in AS-1/5 RBC. No infectious parasites were detected in amustaline–GSH-treated samples after 4 weeks of culture. Amustaline–GSH inactivated high parasite loads regardless of parasite stages and strains. Amustaline readily penetrates the parasite, irreversibly blocks development, and leads to parasite death and expulsion from RBC.

**Discussion:** Amustaline–GSH PRT demonstrated robust efficacy to inactivate malaria parasites in RBC concentrates. This study completes the portfolio of studies demonstrating the efficacy of nucleic acid-targeting PRTs to mitigate TTM risks as previously reported for platelet concentrates, plasma, and WB.

Cissé Sow and Amélie Bouissou are co-first authors.

**Author responsible for reprint requests:** Marion C. Lanteri (mlanteri@cerus.com).

KEYWORDS
Amustaline–glutathione, pathogen reduction technology, *Plasmodium falciparum*, transfusion-transmitted malaria

## 1 | INTRODUCTION

Malaria is caused by the protozoan parasite *Plasmodium* and is transmitted through the bite of *Anopheles* mosquitoes. During its life cycle, *Plasmodium* undergoes asexual reproduction in red blood cells (RBC), which is responsible for the disease symptoms. Malaria is one of the most important parasitic diseases and a great public health problem worldwide with 229 million cases and 409,000 deaths recorded in 2019.[1] The majority of cases were found in African regions and caused by *P. falciparum*, which is responsible for the most severe cases.

Cases of accidental malaria transmission by blood transfusion or organ transplantations are regularly reported in nonendemic countries[2–5] and delayed diagnosis and severe illness in nonimmune recipients are of great concern.[6,7] Blood screening using even the most sensitive nucleic acid testing assays is not sufficiently sensitive to detect as few as 10 parasites per donation, which is sufficient to cause transfusion-transmitted malaria (TTM).[8–10] Antibody screening for donors at risk for malaria is not specific, resulting in excessive deferrals of donors with past infections and not identifying semi-immune carriers.[11] Currently, the approach to prevent TTM in nonendemic countries consists in the deferral of donors, who are either returning from travel to endemic areas or former residents of endemic areas, the latter being more risky.[12,13] With globalization and increased travels to endemic areas, donor loss is increasingly leading to blood shortages for patients chronically transfused in need of phenotype or genotype matched blood.[9] In endemic countries, malaria prevalence is so high that blood donors and recipients are constantly exposed to the parasite. In this context, donor deferrals and blood screening strategies are neither relevant nor practical and may on the contrary lead to operational strain adversely impacting blood availability. Instead, World Health Organization (WHO) recommends the administration of appropriate prophylaxis for all recipients of blood components to prevent symptomatic malaria infection.[14] However, implementation of such recommendations is impractical considering the cost of malaria prophylactic drugs.[15,16]

Thereby, the use of pathogen inactivation for the treatment of blood products could represent a more sustainable option to reduce the TTM risk. Several pathogen reduction technologies (PRT) exist to mitigate the risk of transfusion-transmitted infections. Nucleic acid-targeting photochemical treatments show high efficiency to inactivate pathogens in platelet concentrates and plasma components.[17] Currently three PRT using ultraviolet (UV) light, with or without a photoactive compound, have demonstrated efficiency to inactivate the malaria parasite. The MIRASOL® PRT system uses riboflavin and UVB illumination to cause nucleic acid damage mediated by reactive oxygen species and demonstrated *Plasmodium* inactivation in plasma and platelet components[18] as well as whole blood (WB).[19,20] The INTERCEPT® Blood System for plasma and platelets is based on amotosalen and UVA illumination to modify or cross-link nucleic acids thus irreversibly blocking replication. The latter is the only PRT that is both CE marked and FDA approved.[21] Another method, Theraflex UV, developed for pathogen inactivation in platelets, is solely based on UVC and does not include the addition of a photosensitive compound.[22]

An alternative nucleic acid targeted PRT system for RBC based on a chemical treatment using amustaline and glutathione (GSH) has been developed to overcome the problem of high UV light absorption by RBC when attempting to inactivate pathogens in WB and RBC concentrates and is under clinical evaluation. Amustaline–GSH treatment has been shown to efficiently inactivate various pathogens, including viruses, parasites, and bacteria in RBC,[21,23] and *P. falciparum* in WB units.[24] In the present study, we extended our previous investigations[24,25] to the inactivation of *P. falciparum* in RBC in additive solutions AS-1 (Adsol) or AS-5 (Optisol) and examined the treatment efficiency according to parasite load, stage, and strains from different origins.

## 2 | MATERIALS AND METHODS

### 2.1 | *P. falciparum* culture

*P. falciparum* strains of different origins and susceptibilities (sensitive (s) or resistant (r)) to antimalarial drugs chloroquine (CQ), pyrimethanime (PYR), and mefloquine (MF) were provided from the Unicellular Eukaryote Collection (MNHN, France): FCB1/Colombia (CQr, PYRr, MFs), 3D7 (CQs, MFs, PYRs, derived from the NF54 strain, a Schipol Airport case, The Netherlands), W2/Indochina (CQr, PYRr, MFs), and its DD2 derived clone (CQr, PYRr, MFr). Strains were maintained in

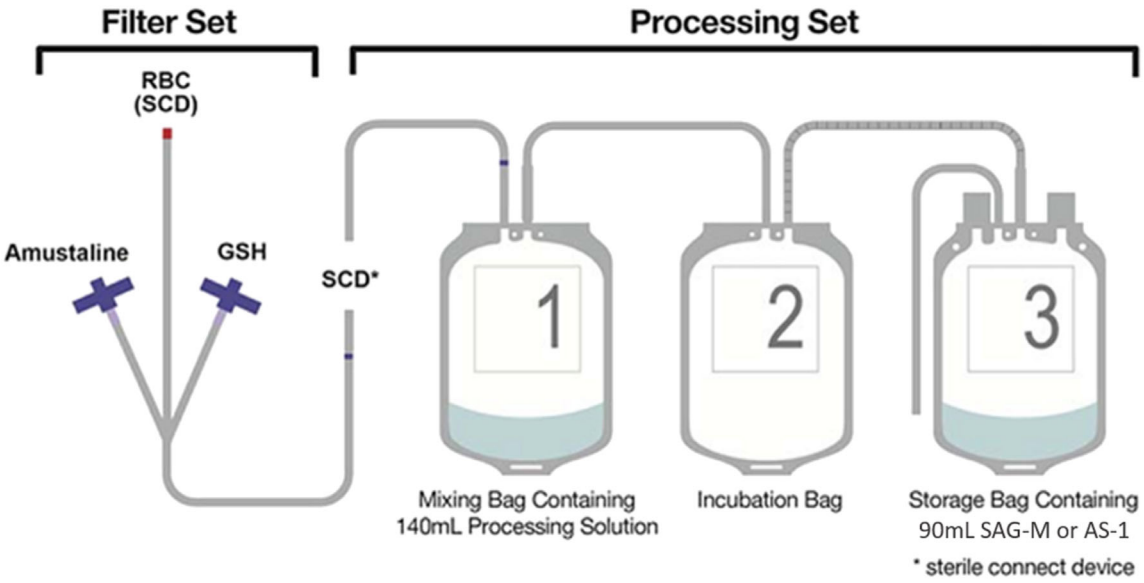


**FIGURE 1** INTERCEPT blood system for RBC. The system comprises an amustaline vial (57 mg amustaline dihydrochloride), a GSH vial (3600 mg GSH), and a single-use trifurcated set with tubing and two 0.2 filters with capped Luer lock fittings. The filter set is connected to a processing set constituted of: one mixing bag, containing 140 ml of processing solution (bag 1, adenine 1.3 mM, mannitol 55 mM, sodium citrate 2-hydrate 20 mM, disodium phosphate 12-hydrate 6.1 mM, monosodium phosphate 1-hydrate 10 mM), one incubation bag (bag 2), and one final storage bag containing 90 ml of either SAG-M or AS-1 additive solutions, depending on the system configuration. Amustaline and GSH vials were reconstituted at the time of the processing with 18.5 ml and 15.5 ml 0.9% sodium chloride solution to give a 6 mM amustaline and a 600 mM GSH solution. The trifurcated set was connected to the RBC unit and to bag 1. GSH solution was first added to bag 1 through one of the 0.2 mm filter ports, and the *P. falciparum*-inoculated RBC was transferred to bag 1 and mixed (final GSH concentration of 20 mM). Amustaline solution was added to bag 2, via the second 0.2-mm filter to a final concentration of 0.2 mM. RBC were transferred to bag 2 and stored at RT up to 24 h. After incubation, bag 2 was then centrifuged to separate the RBC, and the supernatant was expressed into bag 1, which was then sealed off and discarded. Solution from storage bag 3 was transferred to bag 2. After mixing, the content was transferred to bag 3 and stored at 4°C [Color figure can be viewed at wileyonlinelibrary.com]

culture on human RBC (A/O groups) provided by the Etablissement Français du Sang (EFS) in RPMI 1640 medium supplemented with 0.5% Albumax-II, at 37°C, under 5% $CO_2$ as described.[24] Parasites were synchronized by sorbitol treatment[26] and ~20% ring cultures were obtained as described.[27] Parasite development was routinely followed on Diff-Quik-stained smears (Medion Diagnostics). Parasitemia was determined by cytometry using the fluorescent DNA probeYOYO-1 as described.[24,28]

## 2.2 | RBC concentrate collection

Leukocyte-reduced A group RBC suspended at approximately 60% hematocrit in additive saline solution AS-1 (Adsol, Fenwal) or AS-5 (Optisol, Terumo Medical Corporation) were prepared from WB collected with CPD anticoagulant and stored at 4°C. *Plasmodium* inoculation and treatment were performed no more than 2 days postdonation as described below. AS-1 and AS-5 RBC units were adjusted to 360 ml and 280 ml, respectively, prior to amustaline–GSH treatment. A minimum of four independent replicates was performed.

## 2.3 | Amustaline–GSH treatment

For AS-5 RBC, a total of $1.4 \times 10^9$ ring-infected RBC (iRBC) from a culture of ~20% parasitemia were pelleted ($600 \times g$, 5 min), resuspended in 30 ml of RBC withdrawn from the unit, and delivered back and mixed targeting *Plasmodium* levels of $5 \times 10^6$ iRBC/ml in the 280 ml unit. The unit was processed using the INTERCEPT blood system for RBC (Figure 1) as described in Figure 2. Briefly, a 600 mM GSH solution was added to the processing bag followed by the transfer of the contaminated RBC. A sample (17.5 ml) was withdrawn and aliquoted into ventilated 15 ml tubes (2.5 ml per tube) to determine the parasite load at different time points of the process (untreated controls [UT]). Amustaline was then added and after mixing, RBC were transferred to the incubation bag and stored at room temperature (RT). Treated sample (2.5 ml) was withdrawn after 3 h of

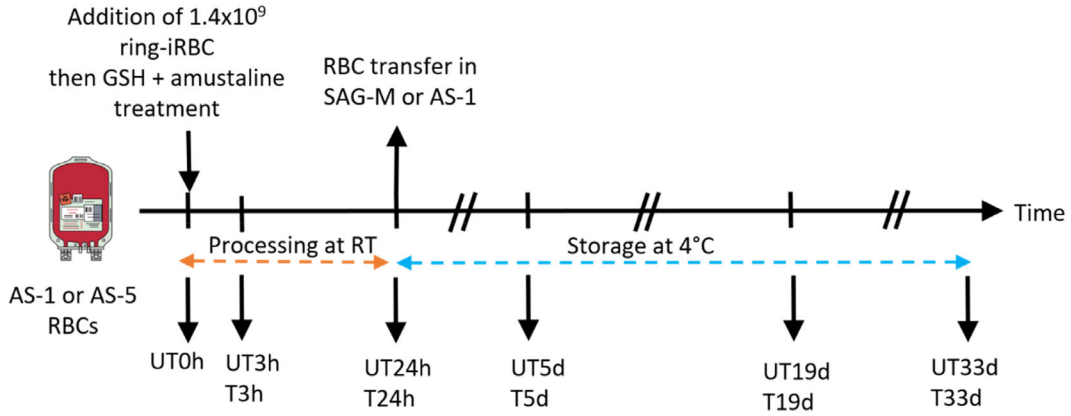


FIGURE 2 Time points of the evaluation of the *P. falciparum* inactivation process in AS-1 or AS-5 RBC concentrate. A AS-1 or AS-5 RBC unit was infected with $1.4 \times 10^9$ ring-infected RBC. Untreated control samples (UT) were removed after addition of GSH and before addition of amustaline, and maintained at RT for 3 or 24 h (UT3h or UT24h) and for some of them further stored at 4°C up to 32 days (UT5d, UT19d, and UT33d). The amustaline-treated RBC unit was maintained 24 h at RT and further stored at 4°C. Treated samples (T) were collected at the same time points as controls. Controls and treated samples were processed for *P. falciparum* viability assay to determine parasite load and reduction [Color figure can be viewed at wileyonlinelibrary.com]

incubation (T3h) and immediately processed to determine the parasite inactivation efficiency. After 24 h, the incubation bag was centrifuged (Heraeus Cryofuge 6600ti, 4200 × g, 20 min, 22°C) and RBC transferred to the final storage bag containing SAG-M additive solution. A 2.5 ml sample was withdrawn constituting the treated sample 24 h (T24h) and processed as for T3h, as well as the controls UT0h, UT3h, or UT24h. The treated RBC unit was stored at 4°C. Samples (2.5 ml) were withdrawn 5, 19, and 33 days after the beginning of the treatment (T5d, T19d, T33d) and processed for viability assays, as well as the UT samples stored at 4°C after a 24 h of incubation at RT (UT5d, UT19d, and UT33d). For AS-1 RBC, the 360 ml units were inoculated with $1.8 \times 10^9$ iRBC ($5 \times 10^6$ iRBC/ml) and processed as for AS-5 RBC except that samples were collected at UT0h, UT24h, and T24h and final storage bag contained AS-1.

## 2.4 | Plasmodium falciparum viability assay

Collected samples were centrifuged (600 × g, 5 min) and RBC resuspended in culture medium to 5% hematocrit. Ten-fold serial dilutions from $10^{-1}$ to $10^{-7}$ were performed with healthy RBC in medium at a 5% hematocrit (Table 1). For UT0h, UT3h, UT24h, and UT5d, dilutions $10^{-3}$ to $10^{-7}$ were maintained in culture in 4 × 30 ml flask for each dilution and dilutions $10^{-1}$–$10^{-4}$ for UT19d and UT33d. For treated samples, dilutions $10^{-1}$ and $10^{-2}$ were maintained in culture. Every 2–3 days, medium was changed, and parasite growth followed over 4 weeks on stained smears. When parasites were detected, parasitemia was quantified by flow cytometry. For negative cultures, flasks were maintained an additional week. At the end of the period, 2 smears per flask were prepared; if no parasites were detected after observation of 40 microscopic fields (100× objective), the flask was considered negative.

## 2.5 | Parasite load, log reduction, and % parasite survival determination

A culture was considered positive when parasitemia reached 1%. Parasite loads were calculated as described[29] and were expressed as the $\log_{10}$ of the median Tissue Culture Infectious Dose ($\log_{10}$ $TCID_{50}$)[24] using the formula:

$$\text{Log}_{10}\,(\text{TCID}_{50}) = -\text{Log10 (dilution of Group 1)} + \text{PD} * \text{Log10 (dilution factor)}$$

$$\text{PD} = \frac{(\%\text{Infected Group 1} \% - 50\%)}{(\%\text{Infected Group 1} \% - \%\text{infected Group 2})}$$

Where, PD = proportionate distribution; Group 1 = experimental group of highest dilution in which ≥50% of the cultures are positive for viable parasites; Group 2 = experimental group of next higher dilution from Group 1.

Log reduction was calculated for each replicate according to the following formula: Log reduction = Log (pre-amustaline titer ÷ post-amustaline titer), where titer is expressed as log $TCID_{50}$/ml. For samples in which no viable *Plasmodium* was detected, log reduction was calculated using the input titer as the limit of inactivation.

**TABLE 1** Sample dilutions and theoretical loads of infected RBC in AS-5 RBC concentrate

| | RBC sample | Resuspended RBC pellet | 10-fold dilutions | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | $10^{-1}$ | $10^{-2}$ | $10^{-3}$ | $10^{-4}$ | $10^{-5}$ | $10^{-6}$ | $10^{-7}$ |
| Assay volume (ml) | 2.5 | 30 | 4 × 30 | 4 × 30 | 4 × 30 | 4 × 30 | 4 × 30 | 4 × 300 | 4 × 300 |
| iRBC number/assay or 30 ml flask | $8 \times 10^6$ | $8 \times 10^6$ | $7.8 \times 10^5$ | $7.8 \times 10^4$ | $7.8 \times 10^3$ | $7.8 \times 10^2$ | 78 | 7.8 | 0.78 |
| iRBC number/ml | $3.2 \times 10^6$ | $2.6 \times 10^5$ | $2.6 \times 10^4$ | $2.6 \times 10^3$ | 260 | 26 | 2.6 | 0.26 | 0.026 |

*Note*: Calculations are based on a 280 ml RBC concentrate unit at 60% hematocrit, an input of $1.4 \times 10^9$ iRBC, and a 2.5 ml sample collected after transfer of the unit to the processing solution containing GSH.

Percentage of parasite survival at 4°C was calculated by the formula: % survival = $10^a \times 100/10^b$, where a = $\log_{10}$ $TCID_{50}$ at 4°C and b = $\log_{10}$ $TCID_{50}$ of the treated sample UT24h.

## 2.6 | Treatment efficiency according to parasitemia, strains, and parasite stages

For parasitemia-dependent inactivation, ring FCB1 culture was adjusted from 0.1 to 10% parasitemia. After centrifugation, RBC (1.2 ml) was resuspended in 1.73 ml of AS-5/processing solution/GSH (690 µl AS-5, 942 µl processing solution, 97.8 µl of 600 mM GSH in saline solution) and distributed in 24-well plates (488 µl/well). Amustaline (3.26 µl of 30 mM amustaline in 0.1 N HCl /well) was added in treated cultures, while an equivalent volume of 0.1 N HCl was added to the control cultures. The plate was incubated for 24 h at RT. After washes, iRBC were maintained in culture and parasite growth was measured on stained smears. For parasite-strain-dependent inactivation, experiments using FCB1, 3D7, W2, and DD2 strains were performed in the same way except that samples (1% parasitemia) were incubated 24 h at RT in 15 ml-ventilated tubes. For parasite-stage dependent inactivation, experiments were performed as above on the FCB1 strain (1% parasitemia), on ring- or trophozoite–schizont-synchronized cultures. The latter stages being more sensitive to temperature changes than the ring stage, parasites were incubated only 3 h at RT with amustaline instead of 24 h. All experiments were performed in triplicate

## 2.7 | Fluorescence microscopy

*P. falciparum* asynchronous culture was incubated for 20 min at RT in AS-5 containing 200 µM amustaline and 20 mM GSH. After two washes, cells were mounted on slides and analyzed by epifluorescence microscopy upon UV excitation. Image acquisition and processing were performed with Metamorph software (Universal Imaging Corporation). The level of autofluorescence of iRBC incubated without amustaline was subtracted from the acquired images.

# 3 | RESULTS

## 3.1 | Sensitivity of the *P. falciparum* viability assay

The viability culture assay theoretically allows for the detection of a single live parasite per sample for the $10^{-7}$ dilution range (Table 1). This level of sensitivity was experimentally confirmed with parasites systematically detected on smears for UT0h at the $10^{-7}$ dilution in the four replicates. There was a positive and reproducible correlation between the parasitemia development in the different dilutions and the time of culture ($R^2$ value >0.95) (data not shown). This indicates that the viability assay used for determining the efficacy of the amustaline–GSH PRT to inactivate *P. falciparum* was highly sensitive and reliable, allowing detection of alive parasite load equivalent to a single parasite per sample.

## 3.2 | *P. falciparum* survives at 4°C in untreated RBC concentrates

*P. falciparum* survival was assessed in AS-5 RBC stored at 4°C for different periods (Figure 2). No significant difference in parasite survival was observed between the time of inoculation of parasites and after a 24 h incubation at RT. For 4°C stored samples, at least 15.3% ± 4.7% and 0.004 ± 0.003% (n = 4) parasites remained alive after 4 days (UT5d) and 18 days (UT19d) of storage, respectively. No parasite survival was observed after 32 days at 4°C (UT33d).

## 3.3 | Amustaline–GSH PRT efficiently inactivates *P. falciparum* ring stage in RBC

AS-1 or AS-5 RBC units were infected with *P. falciparum* at the ring stage and parasitemia of 0.05%–0.1% ($5 \times 10^6$

**TABLE 2** Inactivation of *P. falciparum* in RBC by the amustaline–GSH treatment

| Input | Replicate | Control samples ($Log_{10}$ $TCID_{50}$/ml) | | Treated samples ($TCID_{50}$/ml) | | Log reduction/ml[a] |
|---|---|---|---|---|---|---|
| RBC in AS-5 | | UT0h | UT24h | T3h | T24h | |
| | 1 | 5.5 | 5.3 | 0.0 | 0.0 | >5.5 |
| | 2 | 5.2 | 5.0 | 0.0 | 0.0 | >5.2 |
| | 3 | 5.0 | 6.0 | 0.0 | 0.0 | >5.0 |
| | 4 | 5.2 | 5.9 | 0.0 | 0.0 | >5.2 |
| | Mean ± SD | 5.2 ± 0.2 | 5.5 ± 0.5 | 0.0 | 0.0 | >5.2 ± 0.2 |
| RBC in AS-1 | 1 | 5.0 | 4.9 | nt | 0.0 | >5.0 |
| | 2 | 5.0 | 5.0 | nt | 0.0 | >5.0 |
| | 3 | 5.2 | 4.5 | nt | 0.0 | >5.2 |
| | 4 | 5.5 | 5.5 | nt | 0.0 | >5.5 |
| | Mean ± SD | 5.2 ± 0.2 | 5.0 ± 0.4 | nt | 0.0 | >5.2 ± 0.2 |

Abbreviations: T, test; UT, untreated controls; nd, not tested.

[a]When no viable parasites were detected in test samples, log reduction was calculated using the input load (UT0h) as the limit of inactivation.

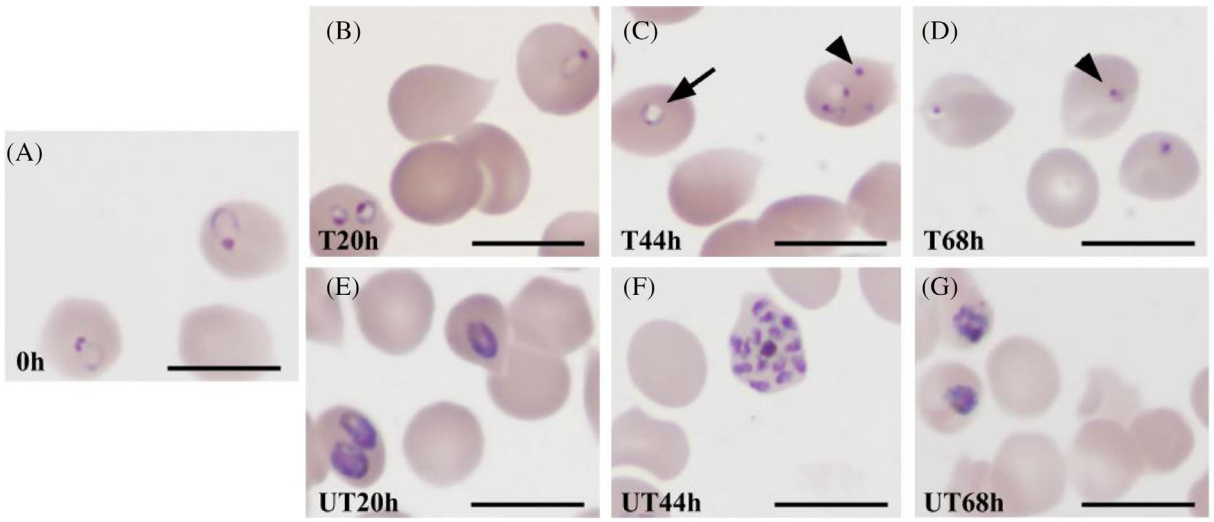


**FIGURE 3** Amustaline treatment effects on *P. falciparum* morphology at 10% parasitemia. **(**A) Ring stage parasites before the 24 h-amustaline treatment at RT; (B-D) treated parasites maintained in culture after treatment; (B) 20 h of culture showing parasites blocked in ring stage; (C) 44 h of culture showing ring stages with an unusual large vacuole (arrow) or in pyknotic forms (arrow head); (D) 68 h of culture showing residual pyknotic parasites (arrow head); (E-G) untreated parasites maintained 24 h at RT and further in culture; (E) 20 h of culture showing ring parasites evolved in trophozoite and schizont stages; (F) 44 h of culture showing parasites evolved in late schizonts full of merozoites, reinvasion occurred with formation of new rings; (G) 68 h of culture showing ring growth into trophozoites. Scale bars for all images represent 10 μm. T = treated, UT = untreated [Color figure can be viewed at wileyonlinelibrary.com]

parasites/ml). For UT0h, the mean experimental parasite load of *P. falciparum* as determined by the viability assay was 5.2 ± 0.2 $log_{10}$ $TCID_{50}$/ml (Table 2). After a 24 h incubation with amustaline–GSH, no evidence of parasite growth was found in the treated samples (T24h) up to 4 weeks of culture. Considering the sensitivity of the parasite viability assay, these data demonstrated complete inactivation of parasites in the collected samples (average log reduction of >5.2 $log_{10}$ $TCID_{50}$/ml). Amustaline has a short half-life of 20 min in aqueous solution,[30,31] a reduction in the length of the standard treatment was investigated for AS-5 RBC (3 h vs. a 24 h-treatment). No viable parasites were detected after 3 h of incubation, supporting the high efficiency of the amustaline–GSH PRT to inactivate *P. falciparum* in AS-1/5 RBC despite the short half-life of native amustaline.

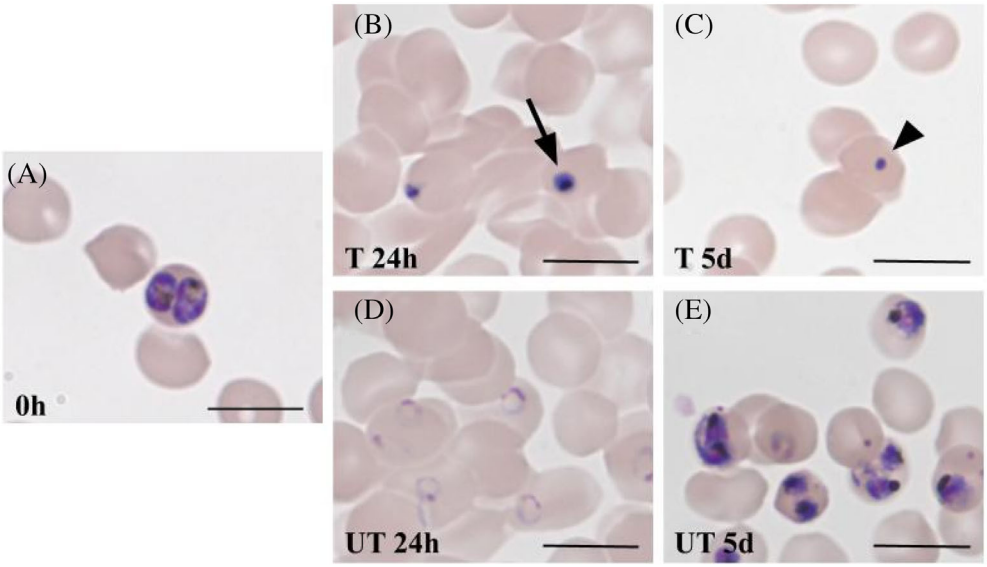


**FIGURE 4** Amustaline effects on trophozoite/schizont stage of *P. falciparum*. (A) Schizont stage parasites before the 3 h-treatment with amustaline at RT; (B) treated parasites maintained 24 h in culture after treatment showing pyknotic parasites (arrow); (C) treated parasites 5 days after treatment showing a residual pyknotic form (arrowhead); (D) untreated parasites maintained 3 h at RT and 24 h in culture, showing new ring stages; (E) untreated parasites maintained 5 days in culture showing trophozoites after 2 complete cycles. Scale bars for all images represent 10 μm. T = treated, UT = untreated [Color figure can be viewed at wileyonlinelibrary.com]

### 3.4 | Treatment-induced parasite growth arrest and led to parasite death

To investigate higher levels of parasitemia (up to 10%), AS-5 RBC infected by rings were treated with amustaline–GSH, maintained in culture and the parasite development monitored. Despite the very high parasite input, no surviving parasites were detected on smears after 5 days of culture even for the highest parasitemia level tested (10%) corroborating the efficiency of the amustaline–GSH treatment to inactivate very high levels of parasitemia.

Figure 3 represents the morphological effects of the treatment on rings. In control RBC, rings evolved into trophozoite–schizonts that further differentiated into merozoites and reinvaded RBC (Figure 3E–G). In contrast, treated rings did not develop into trophozoites. They displayed an unusual large vacuole in the center of the parasite. Two days after treatment, parasites showed intracellular lysis, DNA condensation, became pyknotic (Figure 3B–D), and were progressively expelled from RBC. Treatment efficacy was also investigated for parasites at the trophozoite–schizont stages (1% parasitemia) after a 3 h-treatment incubation. Complete inactivation of parasites was observed. Parasite development was arrested and no RBC invasion was observed. Parasites exhibited a typical pyknotic morphology (Figure 4B,C) and disappeared from the cultures over time. In controls, parasites developed and undertook new intraerythrocytic cycles (Figure 4D,E).

### 3.5 | Treatment is efficient on different strains of *P. falciparum*

It is well-known that *P. falciparum* strains show high variability in terms of resistance to environmental constraints, in particular to xenobiotics. The efficiency of amustaline–GSH PRT was evaluated on the 3D7, W2, and DD2 strains from different geographical origins and having different sensitivities to current antimalarials. Assays were performed at 1% parasitemia at the ring stage under standard treatment conditions. As observed for the FCB1 strain, efficient inactivation of parasites was also observed for the three other strains. After treatment, no surviving parasites were detected on smears after 5 days of culture. Rings became pyknotic and dead parasites disappeared in cultures overtime (data not shown).

### 3.6 | Amustaline readily penetrates intracellular parasites

Amustaline incorporation by *P. falciparum*-iRBC was investigated by fluorescence microscopy taking advantage of the fluorescence properties of its acridine moiety.

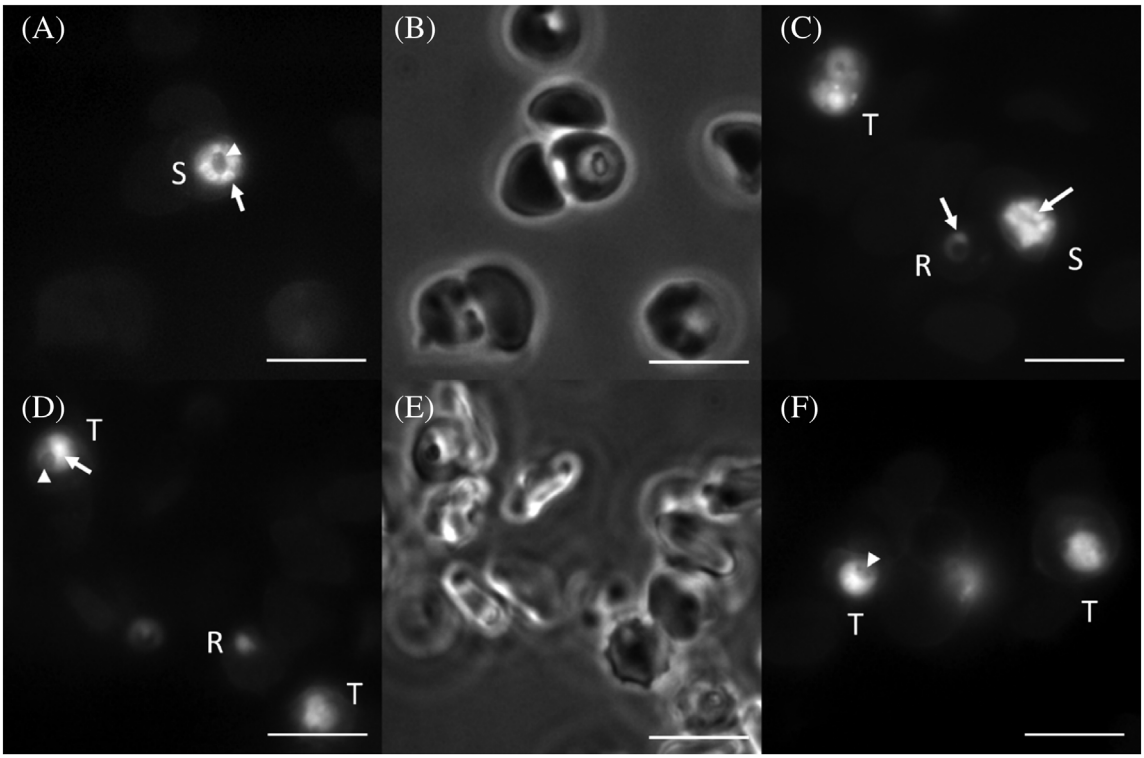


**FIGURE 5** Visualization of amustaline incorporation in *P. falciparum*-infected RBC by fluorescence microscopy. Cells were incubated for 20 min with 200 μM amustaline. (A-B) and (D-E,) fluorescence and the corresponding phase-contrast image. (C and F) fluorescence images. Noninfected RBC were dimly fluorescent. By contrast, a strong fluorescence was associated with any stages of the intracellular parasite: Ring (R), trophozoite (T), schizont (S). Arrowhead: food vacuole. Arrow: intracellular structure strongly stained by amustaline. All scale bars represent 10 μm

Figure 5 shows a panel of representative images of iRBC incubated with amustaline–GSH in AS-5. Noninfected RBC displayed no, or only dim, fluorescent labeling. By contrast, a high fluorescence was associated with the intracellular parasites at all stages. The parasite cytoplasm is labeled with a weaker fluorescence due to the acidic food vacuole (arrowhead). Strongly labeled structures (arrow) were observed, especially in schizonts.

## 4 | DISCUSSION

TTM risk is well documented and current mitigation strategies to ensure blood safety in nonendemic areas include deferrals of blood donors coming from or with recent travel history to malaria-endemic areas. While deferrals have significantly decreased TTM risks, cases continue to be reported. Current blood screening options consist of serology testing using indirect immunofluorescence antibody test, which is not a high throughput assay and requires specialized staff and flow cytometry capacity, and serology testing using ELISA, with sensitivity issues across *Plasmodium* species. Additional challenges include difficulties in detecting chronic carriers with low-level antibodies or semi-immune individuals with a dampened antibody response.[9,32] Asymptomatic donors with parasitemia may go undetected even when a combination of deferrals and screening is in place. Such policies adversely impact blood availability, especially when blood products from highly matched donors are needed for chronically transfused populations. Donor loss is expected to worsen with the increasing worldwide travel and vector range expanding to nonendemic areas under climate changes.[9] Nucleic acid testing assays for the detection of all *Plasmodium* species are under development and may replace serology screening, but they might show limits of detection for low-level parasitemia in RBC unit.[9,32] Therefore, the prospective of robust PRT is appealing to cover both ends of the parasitemia burden.

In endemic areas, where WHO recommendations for donor suitability are difficult to implement, the main strategy is based on the ability of each country to administer malaria prophylaxis for all blood recipients.[15] In such context, PRT represents a promising approach with additional potential benefits beyond TTM risk. In both endemic and nonendemic areas, PRT may increase blood continuity and availability, decrease blood shortages, and replace complex or unpractical donor screening

**TABLE 3** The extent of inactivation ($log_{10}$ reduction) of *P. falciparum* in labile blood products by amotosalen–UVA and amustaline–GSH INTERCEPT PRTs

| Blood labile products | Inactivation system | $Log_{10}$ reduction/ml |
|---|---|---|
| Platelets in 100% plasma | amotosalen/UVA | >6.7 +/− 0.4,[21b] |
| Platelets in 35% plasma/65%PAS | amotosalen/UVA | >6.1 +/− 0.7,[25b] |
| Plasma | amotosalen/UVA | >6.2 +/− 0.4,[25b] |
| RBC in AS-1 | amustaline/GSH | >5.2 +/− 0.2[a] |
| RBC in AS-5 | amustaline/GSH | >5.2 +/− 0.2[a] |
| Whole blood | amustaline/GSH | >5.7 +/− 0.6[24] |

*Note*: a: present study; b: data are the mean +/− the standards deviations of at least 8 replicates from the references cited and unpublished data; Calculation of log reduction was done with the input titer as the limit of inactivation when no viable parasites are detected. ">" refers to inactivation below the limit of detection of the assay.
Abbreviation: PAS, plasma additive solution.

algorithms. Photochemical pathogen reduction treatments are limited by the presence of hemoglobin, which absorbs the light and thus reduces the inactivation efficacy. An alternative nucleic acid-targeting PRT using amustaline–GSH without use of light was developed and is under clinical evaluation. This chemical treatment was previously shown to be highly efficient at inactivating *P. falciparum* in WB[24] and demonstrated its robustness at inactivating a wide range of pathogens, including viruses, bacteria, and parasites in RBC.[30,33–35]

In the present study, the efficiency of the amustaline–GSH PRT to inactivate *P. falciparum* in RBC in AS-1 and AS-5 additive solutions is demonstrated. To reproduce malaria infection generally observed in donors, blood components were inoculated with parasites at the ring stage, the main parasite-stage found in peripheral blood collected for transfusion. Our study demonstrates a robust inactivation of *P. falciparum* upon amustaline–GSH PRT, resulting in a parasite titer reduction of at least 5.2 $log_{10}$ $TCID_{50}$/ml, even for a 360 ml RBC unit, which is the upper limit of the volume guardband for input RBC. The *P. falciparum* viability assay developed for this study was shown to be highly sensitive, allowing detection of a single live parasite per sample, supporting complete parasite inactivation was obtained in the enumerated sample, as no relapse of treated parasites was observed after 4 weeks of culture. Notably, the parasitemia used in the RBC concentrates ($5.6 \times 10^6$ parasites per ml) was within the range of the highest parasite concentrations found in donations in an endemic region of Ghana (50 to $1.87 \times 10^7$ parasites per ml).[20]

The high efficiency of the amustaline–GSH PRT to inactivate *P. falciparum* was corroborated by comparative morphology of treated versus untreated parasites. Untreated parasites normally develop and undergo new intraerythrocytic cycles. In contrast, growth of treated parasites was completely and irreversibly inhibited, and parasites rapidly degenerated becoming pyknotic and were progressively expulsed from RBC. No parasite relapse was observed. Such a pyknotic picture, also called crisis "form", is typical of degenerating forms of the parasite-induced by stresses such as drug pressure, immune response, or increased temperature.[36] Whether the amustaline–GSH-induced parasite death is through apoptotic or nonapoptotic mechanisms (necrosis or autophagy) needs further investigation.

Amustaline–GSH PRT was shown to be highly efficient to inactivate *P. falciparum* strains from different origins and have different resistance phenotypes to current antimalarial drugs. Parasites were effectively inactivated regardless of their development stage, and pathogen inactivation treatment resulted in a similar cell death phenotype, even at very high parasitemia (up to 10%). Taking advantage of amustaline's intrinsic fluorescence, we were able to demonstrate rapid amustaline accumulation inside the live parasites at all stages. No accumulation was observed into noninfected RBC. The entire parasite was visible by fluorescence; however, intracellular structures were particularly marked. Although at this level of observation, resolution does not allow identification of these structures, their shape (round staining), and their number in dividing schizont stages appear to resemble DNA–DAPI (4′,6-diamidino-2-phenylindole) staining, suggesting amustaline accumulation inside the nucleus, which is consistent with amustaline's described mechanism of action: targeting nucleic acids through its acridine moiety and cross-linking of nucleic acids through its bis-alkylator group, thus preventing DNA replication.[30] Considering its mechanism of action and the rapid accumulation of amustaline in infected RBC, it is expected amustaline–GSH PRT will inactivate other *Plasmodium* species reported in TTM as efficiently as it inactivates *P. falciparum*.

Storage at 4°C of RBC has a negative impact on *P. falciparum* survival and is a recommended means to reduce the TTM risks.[37,38] Despite previous observations[31,38] that storage at 4°C effectively reduced parasite survival as a function of storage time, our data show that TTM risk persists through the first 18 days of storage at 4°C. While a very low number of living parasites (0.004%) was measured after 18 days of storage, for a 280 ml RBC unit contaminated with $1.49 \times 10^9$ parasites, an amount of $5.6 \times 10^4$ parasites could remain infectious. It is well established that the minimum infectious dose is very low for *Plasmodium* with as little as 10 parasites being able to transmit infection.[10] All together this study supports the need for further TTM risk mitigation and demonstrates the efficacy of the amustaline–GSH PRT to inactivate the malaria agent in RBC.

In conclusion, the present study complements the comprehensive portfolio of studies documenting the robustness of the INTERCEPT Blood System PRT to inactivate *P. falciparum* in plasma, platelet concentrates resuspended in PAS or 100% plasma, WB, and now RBC concentrates in AS-1 and AS-5 additive solutions (Table 3). Amustaline–GSH, like amotosalen–UVA, provides robust PRT to inactivate malaria parasites to nondetectable levels in various labile blood products for transfusion.

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## CONFLICT OF INTEREST

Yvette A. Girard, Gurvani B. Singh, Marion C. Lanteri, Peter Bringmann are employees of Cerus Corporation, and Jean-Marc Payrat, of Cerus Europe BV. Cissé Sow, Amélie Bouissou, Lotfi Bounaadja, Philippe Grellier, Delphine Haas, Hervé Isola declare no conflict of interest relevant to this manuscript.

## ORCID

*Marion C. Lanteri* https://orcid.org/0000-0002-4213-5013

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