2-picolylamine derivatization for high sensitivity detection of abscisic acid in apicomplexan blood-infecting parasites

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ABSTRACT

We have developed a new liquid chromatography-electrospray ionization tandem mass spectrometry methodology based on 2-picolylamine derivatization and positive ion mode detection for abscisic acid (ABA) identification. The selected derivatization reaction leads to the formation of an amide derivative which contains a highly active pyridyl group. The enhanced ionization allows for a 700-fold increase over commonly monitored underivatized ABA, which in turn leads to excellent limits of detection and quantification values of 0.03 and 0.15 ng mL^{-1}, respectively. This method has been validated in the highly complex matrix of a red blood cell extract. In spite of the high sensitivity achieved, ABA could not be detected in *Plasmodium falciparum*-infected red blood cells, suggesting that, if present, it will be found either in ultratrace amounts or as brief bursts at defined time points within the intraerythrocytic cycle and/or in the form of a biosynthetic analogue.

Keywords: Liquid chromatography-electrospray ionization tandem mass spectrometry; Abscisic acid; Picolylamine; Malaria; *Plasmodium falciparum*; Apicomplexa

1. Introduction

Human-infecting intracellular parasites of the Apicomplexa phylum are responsible for millions of clinical cases resulting in thousands of deaths every year, mainly in developing tropical regions [1]. The Apicomplexa with higher clinical impact can be classified into three principal genera/diseases: *Plasmodium*/malaria, *Babesia*/babesiosis and *Toxoplasma*/toxoplasmosis. All these pathogens share a characteristic organelle, the apicoplast, a non-photosynthetic plastid which has several metabolic pathways absent in humans and therefore is considered an exceptional target for antiparasitic drug research [2]. Isoprenoid biosynthesis operating in plastid-containing parasites has driven special attention because it is essential for the synthesis of numerous downstream metabolites that include ubiquinone, carotenoids and prenylation groups [3]. Carotenoid biosynthesis in the apicoplast has been described in *Plasmodium falciparum* [4] and in *Toxoplasma gondii* [5]. Considering the absence of photosynthetic activity in apicomplexan plastids, diverse alternative roles have been proposed for carotenoid derivatives in these parasites, including, among others [6-8], substrates for the synthesis of plant-like hormones that regulate essential cellular processes [5,9,10]. In this regard, abscisic acid (ABA, Fig. S1) has been proven to be endogenously produced by *T. gondii* through the C_{40}-carotenoid biosynthetic pathway (C_{40}-CBP) [5]. ABA is necessary for *Toxoplasma* reinvasion, regulating the maturation and egress of parasites from the host infected cell in a complex calcium-dependent signaling cascade [3]. The identification in *P. falciparum* of an host cell egress mechanism analogous to that present in *T. gondii*, together with the growth dependence of the parasite on intracellular oscillating calcium levels during most of its intraerythrocytic stage [11], are consistent with the possible existence of ABA or an ABA-like hormone in this pathogen. Accordingly, the herbicide fluridone (Fig. S1), which targets the phytoene desaturase enzyme in the early steps of the C_{40}-CBP, efficiently delayed *T. gondii* growth and prevented its multiplication while significantly reducing ABA levels in the intracellular parasite [5]. Other compounds with an even stronger effect on ABA synthesis, such as the lipoxygenase inhibitor nordihydroguaiaretic acid (NDGA, Fig. S1), have been identified in plants [12] but to date have not been assayed against apicomplexan parasites.

Liquid chromatography-electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS) is currently considered the technique of choice for the determination of ABA and other phytohormones [13-17], providing excellent sensitivity, specificity and

throughput, and permitting the analysis of non-volatile and thermally unstable compounds. Although the sensitivity of LC-ESI-MS/MS (in the range of a few ng mL^{-1}) allows the detection of endogenous levels of ABA in plant material, significantly smaller concentrations of this hormone are expected to be produced by apicomplexan parasites due to the absence of most plant-like, ABA-regulated processes in these pathogens. This calls for the availability of high-sensitivity ABA detection protocols. Several chemical derivatization procedures have been developed in order to enhance the detection responses of carboxylic acid groups, such as the one present in ABA, in LC-ESI-MS/MS [18]. Picolylamine (PA), a derivatizing agent displaying a highly ESI-active pyridyl group that reacts with carboxylic acids in the presence of a condensation agent to form an amide derivative, has been employed for the analysis of fatty acids and other carboxyl-containing compounds [19].

In the present work we propose, for the first time, the use of PA for the derivatization of ABA as the basis of an ultra-high sensitive approach for the detection of this compound in red blood cell-infecting apicomplexan parasites, this being in turn a more efficient protocol for ABA determination than the currently employed gas chromatography-MS assay, which requires the methylation of ABA [5]. In addition to the optimized detection parameters, the results obtained after the application of a full validation protocol for ABA detection in *P. falciparum* are presented.

2. Materials and methods

2.1. Chemicals and reagents

ABA and deuterated ABA ([^{2}H$_6$]ABA or ABA-d6; used as internal standard, ISTD), 2-PA, 2,2'-dipyridyl disulfide (DPDS), triphenylphosphine (TPP) and saponin were purchased from Sigma-Aldrich Corporation (St. Louis, MO, USA). Ethyl acetate, methanol (LC gradient grade), formic acid and ammonium formate (LC/MS grade) were obtained from Merck (Darmstadt, Germany). Ultrapure water was obtained from a Milli-Q purification system (Millipore Ibérica, Barcelona, Spain).

2.2. ABA purification from cell extracts and derivatization

2.2.1. Liquid-liquid extraction (LLE)

An aliquot of 150 µL of the lyophilized cell extract (Supplementary material) previously re-dissolved in 1 mL of acetonitrile:water (8:2 v/v) was supplemented with 25

µL of 1 ng mL^{-1} ABA-d6 and the mixture was evaporated under N$_2$ stream at 40 °C. After addition of 1 mL of a saturated NaCl solution and 1 mL of 1 M acetic acid, the mixture was extracted with 6 mL of ethylacetate by shaking in a rocking mixer at 40 oscillations min^{-1} during 20 min. After centrifugation (3000 g, 5 min), the organic phase rich in protonated ABA was separated and evaporated to dryness under a N$_2$ stream coupled to a 40 °C water bath.

2.2.2. Solid phase extraction (SPE)

Alternatively to LLE, the lyophilized cell extracts re-dissolved in 1 mL of acetonitrile:water (8:2 v/v) were processed by SPE. The mixture of re-dissolved extract and ABA-d6 was vortexed and passed through a Sep-Pak® Vac RC (500 mg) C18 cartridge (Waters Associates, Milford, MA, USA) previously conditioned with MeOH and washed with deionized water (2 mL of each). The column was rinsed with 2 mL deionized water and ABA was eluted with 2 mL MeOH. The methanolic extract was evaporated to dryness under a N$_2$ stream in a 40 °C water bath.

2.2.3. ABA derivatization with 2-PA

The resulting dry pellet from either LLE or SPE was derivatized based on the procedure described by Higashi et $al.$ [18]. Briefly, 10 µL each of freshly prepared 10 mM TPP, 10 mM DPDS, and 1 mg mL^{-1} 2-PA solutions in acetonitrile were successively added to the pellet. The mixture obtained was incubated at 60 °C for 10 min, and after removal of the solvent by evaporation under a N$_2$ stream at 40 °C, the product was reconstituted with 150 µL of deionized water:acetonitrile (9:1 v/v) and 10 µL of the resulting solution were injected into the LC-MS/MS system.

2.3. LC-ESI-MS/MS

PA-derivatized ABA (ABA-PA) was quantified by LC-ESI-MS/MS with a selected reaction monitoring (SRM) method using a triple quadrupole (Quattro Premier XE) mass spectrometer equipped with an orthogonal Z-spray-electrospray ionization source (Waters Associates) interfaced to an Acquity UPLC system (Waters Associates) for the chromatographic separation. Nitrogen was employed as both drying and nebulising gas. The desolvation gas flow was set to approximately 1200 L h^{-1} and the cone gas flow to 50 L h^{-1}. A cone voltage of 25 V and a capillary voltage of 3.0 kV were used in positive ionization mode. The nitrogen desolvation temperature was set at 450 °C and the source temperature at 120 °C. The LC separation was performed using an Acquity

UPLC BEH C18 column (100 × 2.1 mm i.d., 1.8 µm, Waters Associates) at a flow rate of 300 µL min^{-1}. Water and methanol, both containing 0.01% formic acid and 1 mM ammonium formate, were selected as mobile phase solvents. A gradient program was used where the percentage of organic solvent was linearly changed as follows: 0 min, 30%; 0.5 min, 30%; 5 min, 70%; 5.5 min, 90%; 6 min, 30%; 7.5 min, 30%. ABA-PA and ABA-d6-PA (PA-derivatized ISTD) were determined by SRM considering three transitions for each compound.

2.4. Method validation for ABA detection and quantification

The samples selected for validation were processed through LLE plus PA-derivatization (see Methods section 2.2) and finally analyzed by means of the LC-ESI-MS/MS system described in Methods section 2.3. In each of three validation assays, precision and accuracy of the method were checked by running quality control samples (QCs) prepared by spiking lyophilized red blood cell (RBC) extracts with two different concentrations of ABA (0.15 and 1.5 ng mL^{-1}). Calibration standards were analyzed and calibration curves were calculated by least-squares linear regression. Six replicates were done for each level to evaluate intra-assay reproducibility and accuracy, which were expressed, respectively, as the relative standard deviation of the estimated concentrations obtained for the six QCs analyzed in one assay, and as the recovery value (%) estimated for the QCs. Inter-assay reproducibility and accuracy were evaluated similarly from all replicates analyzed in three validation assays by two different operators (n = 12). Limits of detection (LOD) and quantification (LOQ) were defined, respectively, as the lowest concentration displaying a chromatogram signal/noise ratio (S/N) value of 3, and as the concentration which gives a S/N value of 10. ABA concentration for the determination of LOD and LOQ was calculated considering the lyophilized RBC extract resuspended in 1 mL acetonitrile:water (8:2 v/v), as described in Methods section 2.2, which results in approximately 1.2×10^{10} RBCs mL^{-1}.

2.5. Evaluation of the RBC matrix effect

Six blank samples corresponding to 150-µL replicates of a re-dissolved RBC extract were processed by LLE and the resulting dry pellet was subsequently supplemented with 150 µL of 5 ng mL^{-1} ABA and 25 µL of 1 ng mL^{-1} ABA-d6. The same amounts of ABA and ABA-d6 were added in parallel to three clean tubes. The

derivatization reaction with PA was thereafter performed in all nine tubes. Matrix effect in both ABA and ABA-d6 was calculated by means of the comparison between the responses of the RBC extract blank samples spiked after LLE and the solvent standards.

3. Results

3.1. Optimization of the method for ABA detection

The mass spectrometric parameters for PA-derivatized ABA (ABA-PA and ABA-d6-PA ISTD) were optimized in order to achieve an increased signal (Table 1). ABA derivatization (Fig. 1A) proved to notably increase the MS/MS response towards this metabolite when compared to the underivatized species, an effect being illustrated by the chromatograms corresponding to the analysis of the same amounts of ABA (Fig. 1B) and ABA-PA (Fig. 1C). Remarkably, the intensity of the most abundant transition increased from 120 to 92,461 counts after ABA conjugation with PA.

For the optimization of chromatographic conditions, a C18 column typically used for the retention of low polar compounds was chosen. The use of methanol as organic solvent and the addition of low amounts of both formic acid and ammonium formate to the mobile phases were selected in order to promote the protonation in ESI+ [20]. Using a generic gradient from 5% to 95% of organic solvent, it could be deduced that ABA-PA eluted with c.a. 50% of organic solvent. In order to improve the chromatographic resolution, a slower gradient between 30% and 70% of organic solvent was finally selected.

During the development of the method, the presence of ABA was identified in the plant-derived hemolytic agent saponin, which was initially used for the preparation of RBC extracts employed later for the method validation. The unequivocal presence of ABA in saponin-containing solutions was determined by monitoring three different ion transitions of ABA-PA (Fig. S2). To avoid potential contaminations by plant ABA, an alternative saponin-free protocol based on acetone as organic solvent was established and used in this work for the extraction of RBCs.

3.2. Method validation: ABA extraction recovery and RBC matrix effects

Both SPE and LLE were studied as clean-up and pre-concentration steps. Obtained recoveries were 36±18% and 86±13% for SPE and LLE respectively. The

relatively large amount of organic solvent in the extract might increase the breakthrough of the analyte being behind the low extraction recoveries obtained for SPE. A satisfactory linearity was achieved by the method with a correlation coefficient of r = 0.998 for the studied range (0.05-10 ng ABA-PA mL^{-1}). Because electrospray ionization is particularly prone to matrix effects, a specific study concerning this parameter was conducted by comparing the signals of a derivatized standard with and without the presence of the matrix (n = 6). The results proved that the multiple compounds present in a complex sample such as RBC extracts, in this case do not produce significant ionization enhancement or suppression, neither for ABA (89±11%) nor for its ISTD (95±12%). The chromatograms corresponding to the analysis of a blank RBC sample extract (1.2 × 10^{10} cells mL^{-1}) either alone or spiked with 0.15 ng/mL of ABA are presented in Fig. 2.

As result of the ionization improvement provided by the PA-derivative formed, the limits of detection and quantification were respectively reduced to 0.03 and 0.15 ng ABA mL^{-1}, using a RBC-based matrix of 1.2 × 10^{10} extracted cells mL^{-1}. Up to date, these results validate this method as the most sensitive for ABA detection and quantification, despite using as matrix a very large amount of RBC extract. Furthermore, intra-day and inter-day accuracy at both tested concentrations (0.15 and 1.5 ng ABA mL^{-1}) provided excellent results with recovery values higher than 90% (Table S1). Finally, the validation results also showed the excellence of the method in terms of precision, with relative standard deviation values ≤10% in all cases. Because of the high ABA extraction recovery yield obtained, LLE was selected as the method of choice for ABA detection and quantification in *P. falciparum* cultures.

3.3. Analysis of the possible effect of C$_{40}$-CBP inhibitors on ABA synthesis in P. falciparum cultures

The effect on the malaria parasite growth of two compounds known to interfere with the C$_{40}$-CBP, and therefore directly affecting the production of downstream carotenoids as precursor molecules for a potential ABA synthesis, was analyzed in *P. falciparum in vitro* cultures. Both fluridone and NDGA displayed activity against *P. falciparum* intraerythrocytic growth, with respective 50% growth inhibitory concentrations (IC50) of 112.2 and 46.8 µM (Figs. S3 and S4). The validated PA-based, LC-ESI-MS/MS method for ABA detection and quantification was applied to non-infected RBCs and to *P. falciparum* cultures in the trophozoite/schizont maturation stages. Parasitized RBC (pRBC) cultures collected at t$_{24h}$ from the untreated control

sample in growth inhibition assays were extracted with the lyophilization/acetone method (see Supplementary material), subsequently purified by LLE plus PA derivatization and finally analyzed by LC-ESI-MS/MS (ISTD retention time of about 3.3-3.5 min and 355 → 229 m/z as major ABA-PA ion transition). No peak was detected matching the aforementioned conditions (Fig. S5), indicating either the absence of ABA in pRBC extracts or its presence in amounts below the LOD of 0.03 ng mL^{-1}.

In pRBC extracts from growth inhibition assays after 24 h of incubation in the presence of fluridone and NDGA at their previously determined IC50s, an unknown compound was detected having the same molecular mass as ABA-PA and eluting from the liquid chromatography column at the ABA-d6-PA ISTD characteristic retention time (3.3 to 3.5 min). Although this molecule displayed only the 355 → 109 m/z ion transition among the previously determined ABA-PA specific 355 → 229/135/109 m/z major ion transitions (Table 1), it exhibited the predicted ABA behavior after treatment with C$_{40}$-CBP inhibitors, presenting its maximum level in untreated *P. falciparum* cultures but being significantly reduced in the presence of fluridone and NDGA at their respective IC50s (Fig. 3).

4. Discussion

Several analytical approaches previously used for the quantification of ABA include ultraviolet or MS detection, either combined with high performance liquid chromatography [21] or capillary electrophoresis [22]. Initial MS methods were based on gas chromatography coupled to mass spectrometry [5,23-25], with the highest reported sensitivity obtained by forming the pentafluorobenzyl ester of ABA and subsequently detecting this derivative in an electron capture negative ionization source (ion trap mass spectrometer [24], or a quadrupolar instrument [23]). However, this technique is not well suited for the analysis of non-volatile substances or temperature-sensitive compounds and is less sensitive than more recent methodologies currently used for the detection of plant-derived ABA such as LC-ESI-MS/MS. Following the latter approach and working in negative ion mode, excellent detection and quantification limits have been reported ranging from 0.3 to 4 ng g^{-1} dry weight [26-28].

A further improvement in the detection of compounds bearing carboxylic acids had been achieved by means of their conjugation with PA [19], allowing in this manner for a more sensitive detection through positive ion mode; however, this strategy has never been explored for the analysis of ABA. We have successfully combined in this work an efficient LC-ESI-MS/MS system with ABA-PA derivatization, a conjugation step

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enabling ABA to be quantified through selected reaction monitoring in positive ionization mode and ultimately resulting in a highly sensitive method with extraordinarily low LOD and LOQ respective values of 0.03 and 0.15 ng ABA mL^{-1}, with the additional difficulty of employing a complex 1.2×10^{10} RBC mL^{-1} extract as matrix. In the most sensitive determination to date using an acetone-based cell extraction and in which ABA was quantified through the negative ionization mode [26], the respective LOD and LOQ were 0.2 and 0.6 ng g^{-1} dry mass, ca. 7- and 4-fold higher, respectively, than those reported here. Such improvement in ABA detection sensitivity might be crucial for the analysis of this hormone in organisms presenting carotenoid biosynthetic routes analogous to those found in plants, such as parasites of the phylum Apicomplexa including *Plasmodium* and *Babesia*.

ABA is commonly quantified in plant extracts where sample availability is not a limitation. However, its determination in minute amounts from complex biological samples such as within intraerythrocytic apicomplexan pathogens producing carotenoids but lacking most of the ABA-controlled functions found in plants, requires a detailed optimization of the ABA chromatographic and mass spectrometric parameters. After choosing the right conditions for ABA purification and detection, we applied a full validation protocol using human RBCs as matrix in order to better characterize the newly developed method. The results presented here show that our optimized methodology is adequate for its intended purpose in terms of accuracy, precision and linearity.

Some of the reasons that might account for the failure in detecting ABA in *P. falciparum* cultures include: (i) its presence in a concentration below the limit of detection; (ii) the synthesis of this hormone only at a precise time of parasite maturation, such as the moment prior to egress from the host RBC [5], and thereby remaining below detectable amounts during most of the parasite's intraerythrocytic cycle; and (iii) the simultaneous existence of ABA derivatives having different molecular masses or showing different chromatography retention times. The amphiphilic properties of the molecule reflected in its high octanol/water partition coefficient *P* of 123.03 units [29] indicate that at physiological pH ≥ 99.5% of ABA molecules will be in deprotonated (charged) form (Fig. S6), possibly leading to a widespread distribution of ABA throughout the parasitized cell and the extracellular environment. This might eventually result in too low ABA amounts in pRBCs, below the LC-ESI-MS/MS detection level reported here of 0.03 ng mL^{-1}.

Further research is required in order to ascertain if the detected unidentified compound whose levels are affected by ABA biosynthesis inhibitors is actually a

modified derivative of ABA or an interfering unrelated compound generating the ABA-PA-like 355 m/z precursor ion. The application of open metabolomic approaches would be of great interest in order to unveil the identity of such potential ABA analogs, whose existence could significantly contribute to understanding the *P. falciparum* biological processes affected by C_{40}-CBP inhibitors, which would in turn be of paramount importance for the development of future new antimalarial therapies.

5. Conclusion

A highly sensitive LC-ESI-MS/MS approach has been developed for ABA detection, with respective LOD/LOQ values of 0.03/0.15 ng mL^{-1}, which has been validated in RBC matrices and finally applied to late-form pRBC extracts. Nevertheless, the presence of ABA in the human malaria parasite *P. falciparum* remains still unresolved, although inhibitors such as NDGA affecting enzymes directly involved in its biosynthesis have been shown to arrest *Plasmodium* intraerythrocytic growth. Some likely explanations for such dichotomy are the synthesis of ABA by the intracellular pathogen in undetectable amounts, its production only at specific time points of the parasite's life-cycle, or the presence of a chemical derivative with different molecular mass and physicochemical properties. In this regard, further research is mandatory in order to finally resolve if *P. falciparum* and other Apicomplexa besides *T. gondii* are capable of synthesizing ABA. Such discovery would provide novel parasite-specific target pathways for the design of new drugs and improved therapies against *Plasmodium* and *Babesia*. Finally, the optimized method for ABA detection presented here could be applied as well to the study of this hormone in *T. gondii*.

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Figure legends
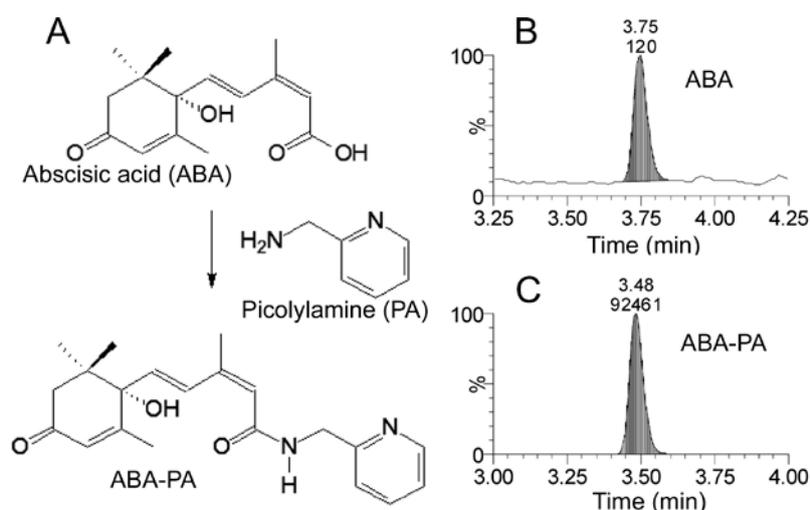
Fig. 1. ABA derivatization with PA and detection of ABA-PA vs. underivatized ABA by LC-ESI-MS/MS operating in SRM mode. (A) Derivatization reaction, (B) ABA detection in a solvent standard at 10 ng mL^{-1} using the ion transition 263 → 153 as optimized in [31] (retention time 3.75 min; peak integration 120 area units), and (C) ABA-PA detection in a solvent standard at 10 ng mL^{-1} using the 355 → 229 major ion transition (retention time 3.48 min; peak integration 92,461 area units).
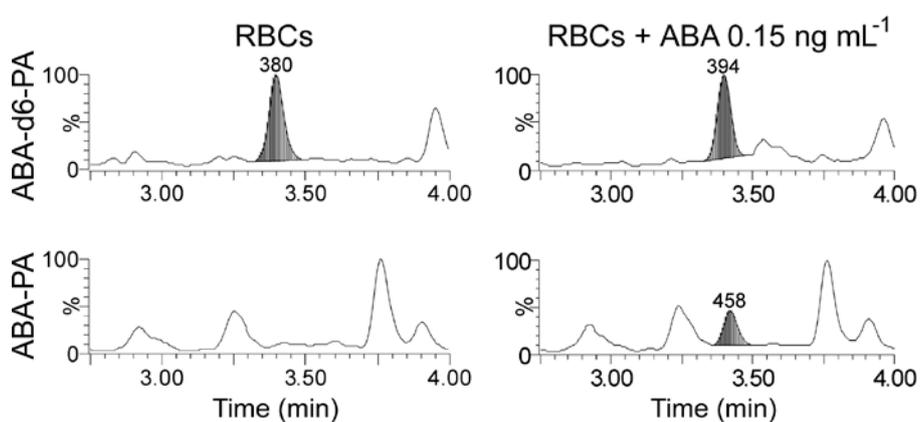
Fig. 2. LC-ESI-MS/MS chromatograms corresponding to ABA-PA and ABA-d6-PA (ISTD) for RBC blank samples (1.2 × 10^{10} extracted cells mL^{-1}) either alone or spiked with 0.15 ng ABA mL^{-1}. Peaks obtained at the characteristic ABA-PA 355 → 229 m/z and ISTD 361 → 235 m/z major ion transitions and detected at the specific retention time are shadowed.
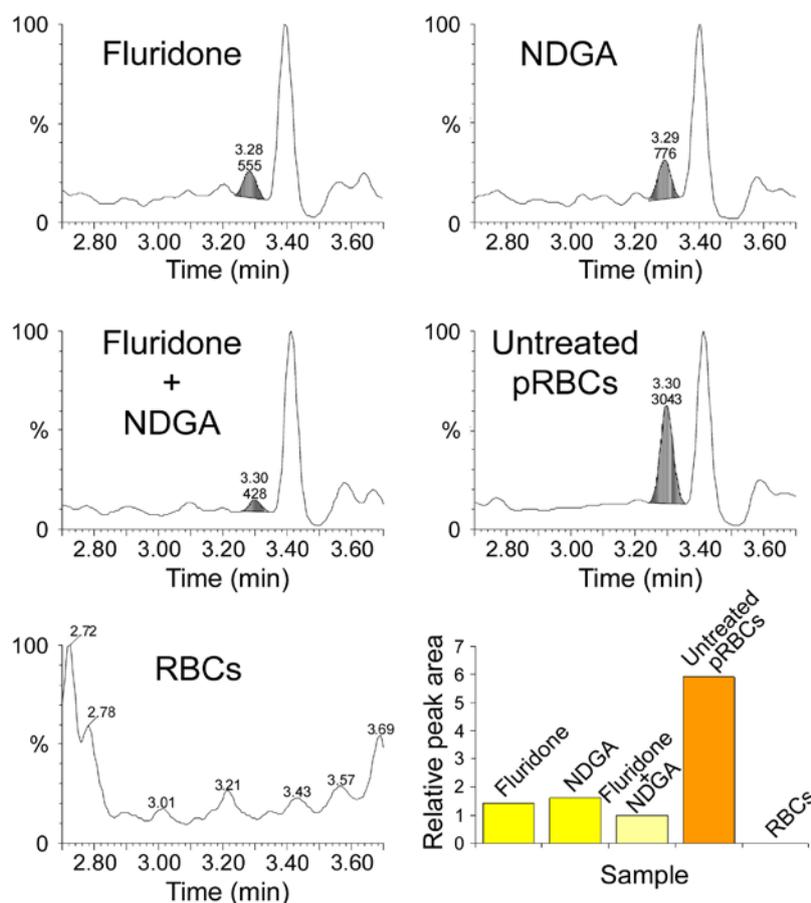
Fig. 3. Unknown compound detected in *P. falciparum* samples treated with C_{40}-CBP inhibitors and exhibiting the hypothesized behavior of ABA. LC-ESI-MS/MS chromatograms corresponding to the total cell extracts obtained from cultures treated with 112 μM fluridone, 47 μM NDGA, and with both drugs combined at the same respective concentrations, from untreated pRBCs, and from non-infected RBCs as negative control. The bottom right panel shows the corresponding areas under the shadowed peak detected at the ISTD retention time of 3.3 min and following the 355 →109 m/z ion transition.

Supplementary material

2-picolylamine derivatization for high sensitivity detection of abscisic acid in apicomplexan blood-infecting parasites

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Supplementary methods

Materials

Except where otherwise indicated, reagents were purchased from Merck & Co., Inc. (Kenilworth, NJ, USA), and reactions were performed at room temperature (22 to 24 °C).

Plasmodium falciparum in vitro culture and growth inhibition assays

The 3D7 *P. falciparum* strain was grown *in vitro* in group B human erythrocytes using previously described conditions [1]. Parasites (thawed from glycerol stocks) were cultured at 37 °C in Petri dishes with red blood cells (RBCs) at 3% hematocrit in Roswell Park Memorial Institute (RPMI) complete medium containing Albumax II (RPMI-A, Invitrogen), supplemented with 2 mM L-glutamine and under a gas mixture of 92% N_2, 5% CO_2, and 3% O_2. For culture maintenance, parasitemia was kept below 5% late forms and 10% early forms by dilution with freshly washed RBCs and the medium was changed every 1-2 days. Synchronized cultures in early ring stages (0-24 h post-invasion) were obtained by 5% sorbitol lysis [2]. Late-form trophozoite and schizont stages (24-36 h and 36-48 h post-invasion, respectively) were purified in 70% Percoll (GE Healthcare) [2,3]. Parasitemia was determined by microscopic counting of blood smears fixed briefly with methanol and stained for 10 min with Giemsa (Merck Chemicals) diluted 1:10 in Sorenson's buffer, pH 7.2. Slides were observed with a Nikon Eclipse 50i (Japan) optical microscope and pictures were taken with a Nikon Digital Sight DS-U2 camera.

For *P. falciparum in vitro* growth inhibition assays, cultures synchronized (>95%) in the early ring maturation stage were brought to 6% hematocrit and 1% parasitemia by dilution with fresh RBCs. After adding one culture volume of 2× concentrated drug solution in RPMI-A to achieve 3% hematocrit, cultures were seeded in 96-well plates (Merck Chemicals) and incubated for a complete 48 h growth cycle under the conditions described above. For growth inhibition determination, samples were processed and analyzed by flow cytometry as described elsewhere [4]. Briefly, cultures were diluted 1:100 in 1× phosphate buffered saline (PBS) and the nuclei of *P. falciparum*-infected RBCs (pRBCs, the only nucleated cells present in the culture) were stained by addition of 0.1 µM Syto11 (Thermo Fisher Scientific, Inc.) in the final mixture before proceeding to their analysis by a BD LSRFortessa flow cytometer (Becton, Dickinson and Company, NJ, USA).

Preparation of C$_{40}$-CBP inhibitor-treated P. falciparum cultures for ABA analysis

P. falciparum cultures at 6% parasitemia and 3% hematocrit synchronized in ring stages were washed several times with RPMI-A and cultured in the presence of C$_{40}$-carotenoid biosynthetic pathway (C$_{40}$-CBP) inhibitors for 24 h until appearance of pRBCs at the trophozoite and schizont late forms. Fluridone and nordihydroguaiaretic acid (NDGA) were assayed at their respective previously determined *in vitro* IC50 (112.2 µM and 46.8 µM), separately and in combination. The same volume of methanol, used as solvent for both inhibitors, was tested in parallel as control (0.1% v/v methanol/culture). An additional control containing only uninfected RBCs at 3% hematocrit was also included. After the 24 h incubation period in the presence of C$_{40}$-CBP inhibitors, *P. falciparum* cultures were spun down and the cell pellets were extracted through the lyophilization/acetone method (see below) as an adaptation of already established protocols for ABA purification [5-7]. In parallel to cell extraction, small aliquots of cultures in trophozoite stage were diluted 7.5-fold in RPMI, supplemented with new RBCs to obtain 3% hematocrit, and further incubated for an additional 24 h in order to determine the parasite reinvasion capacity after treatment.

Lyophilization/acetone cell extraction method

Pelleted cells (1.2 × 10^{10} total cells) contained throughout the whole extraction process in freezing-suitable polypropylene tubes were directly frozen in dry ice/ethanol and lyophilized overnight. The resulting pellets were thereafter weighted and solubilized in 8 volumes of ice-cold extraction solution (80:19:1 acetone:water:acetic acid). Samples were homogenized by vortex, incubated for 15 min and spun down (10,000 *g*, 10 min, 4 °C). The supernatant obtained was stored at 4 °C and the remaining pellet was solubilized again in 8 volumes of ice-cold extraction solution, repeating the procedure two more times. All three supernatants were subsequently pooled and dried, first under N$_2$ flow and finally lyophilized for a complete removal of solvent.

Supplementary results

Effect of inhibitors of the C_{40}-CBP on P. falciparum growth

The effect on the malaria parasite growth of two compounds known to interfere with the C_{40}-CBP, and therefore directly affecting the production of downstream carotenoids as precursor molecules for a potential ABA synthesis, was analyzed in *P. falciparum in vitro* cultures. Both fluridone and NDGA displayed activity against *P. falciparum* intraerythrocytic growth, with respective 50% growth inhibitory concentrations (IC50) of 112.2 and 46.8 µM (Figs. S3 and S4), in accordance with other C_{40}-CBP inhibitors tested in *P. falciparum* and *T. gondii* [5,8]. In growth inhibition assays, fluridone- and NDGA-treated *P. falciparum* cultures displayed a significant developmental delay when compared to untreated controls (Fig. S4), as illustrated by an increase in the number of parasites exhibiting a picnotic nucleus (phenotype indicative of cell death) after 24 h incubation in the presence of either inhibitor, a feature which was exacerbated when parasites were treated simultaneously with both drugs. At the end of growth inhibition assays, 48 h after treatment started, a reduction of the number of parasites in ring early maturation forms could be observed; in addition, treated samples contained late stages prior to egression in significantly larger numbers than untreated control samples. These results strongly suggested a significant effect of fluridone and NDGA in impairing parasite development and replication. Because the observed *P. falciparum* growth-delayed phenotype was analogous to that of the ABA-producing *T. gondii* parasite, and considering the already known activity of fluridone and NDGA as inhibitors of ABA biosynthesis, we proceeded to the optimization of ABA detection and the design of a validation method to be later applied to *P. falciparum* culture cell extracts.

Supplementary figures
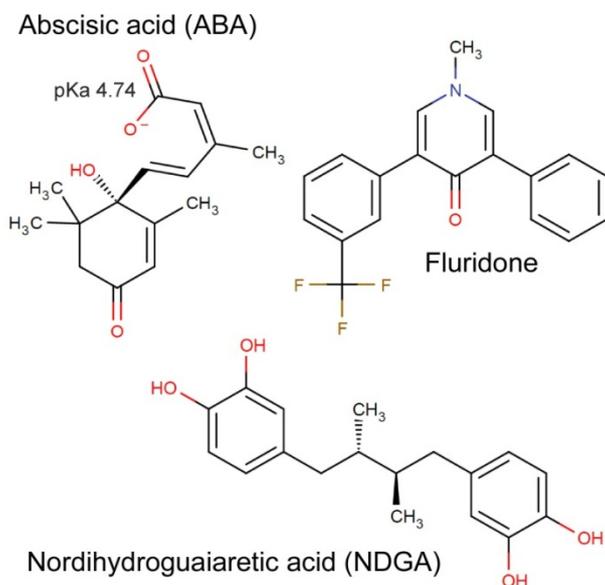
Fig. S1. Molecular structures of ABA, fluridone, and NDGA. From [9].
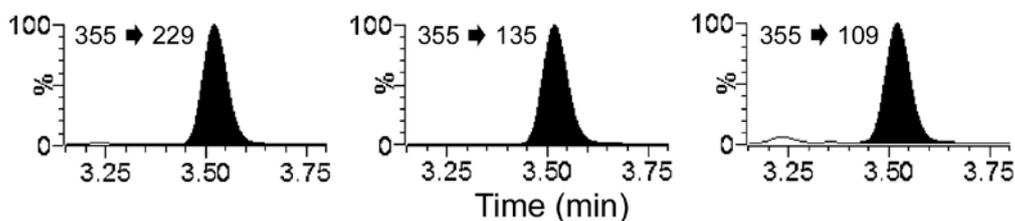
Fig. S2. Detection of ABA in a 0.15% v/v saponin solution in PBS. LC-ESI-MS/MS chromatograms show the three major ion transitions (m/z) of ABA-PA.
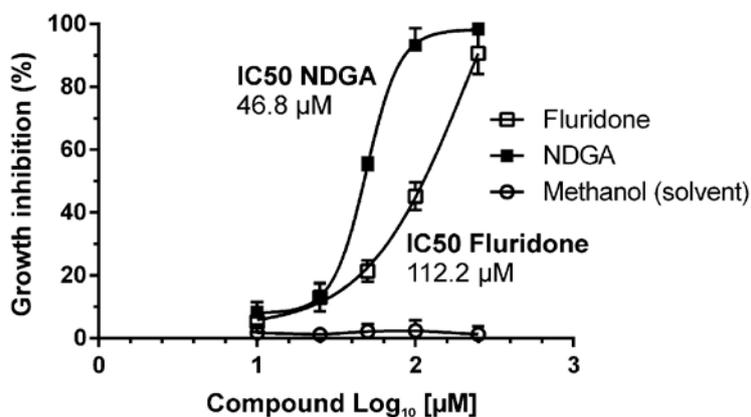
Fig. S3. *P. falciparum* growth inhibition assays of the effect of fluridone and NDGA added to ring stage cultures and analyzed after one replication cycle (48 h incubation). <mark>Bars indicate standard deviations.</mark>
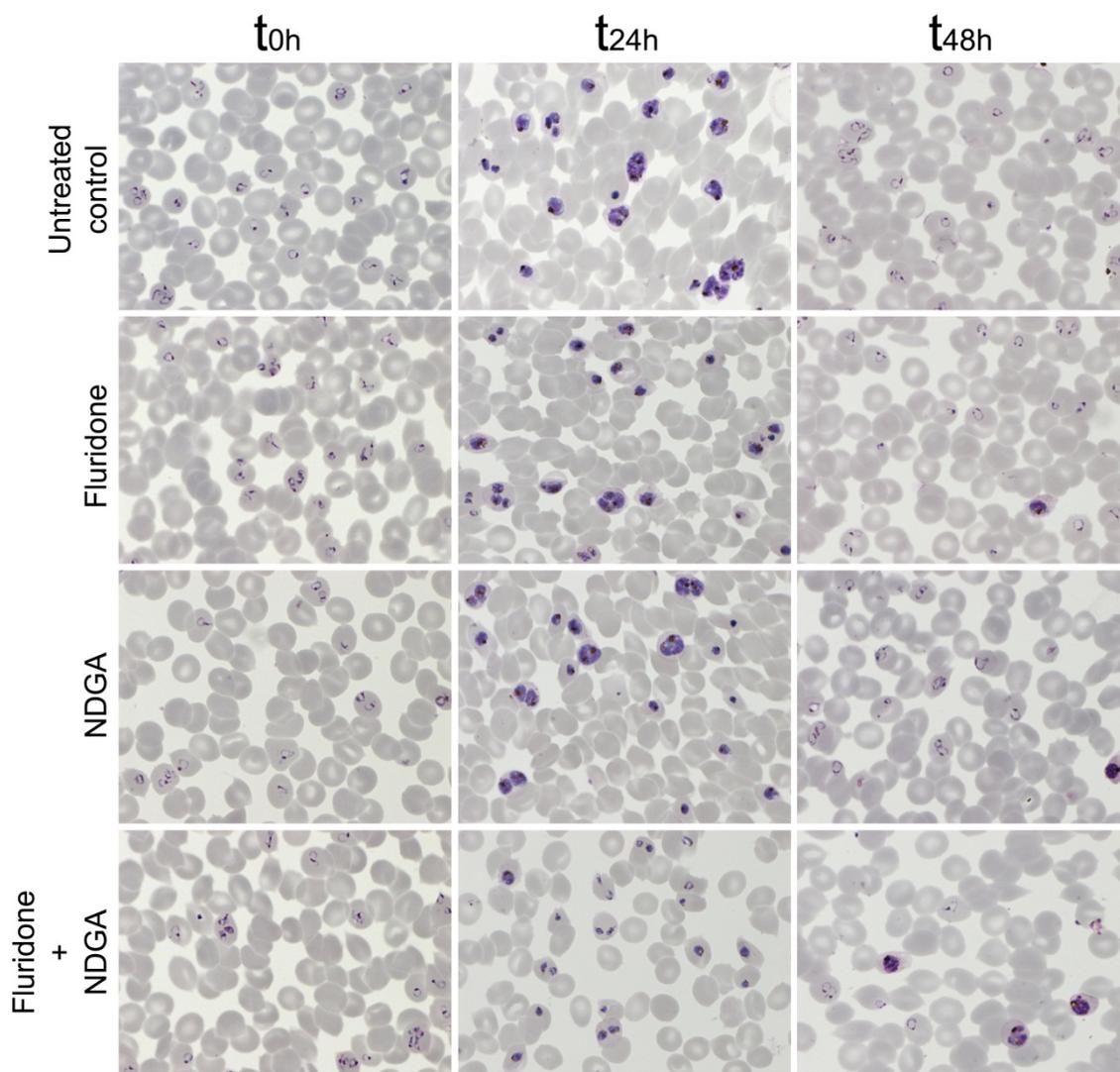
Fig. S4. Microscopic images of Giemsa-stained *P. falciparum* cultures corresponding to growth inhibition assays in the presence of fluridone and NDGA tested at their respective IC50s determined in the assay presented in Fig. S3. Images were obtained at t_{0h} (parasites at ring stage), t_{24h} (trophozoite and schizont stages), and t_{48h} (ring stages again in untreated samples). Parasites collected at t_{24h} samples were employed later for ABA determination in *P. falciparum* cultures.
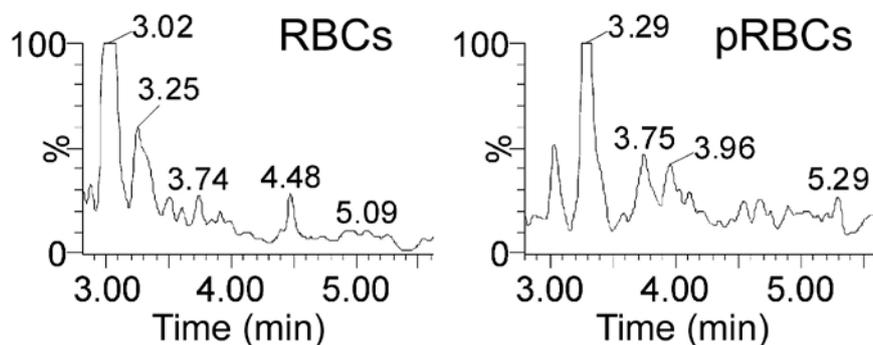
Fig. S5. LC-ESI-MS/MS chromatograms corresponding to ABA analysis in RBC and pRBC cell extracts obtained from *P. falciparum* untreated cultures at the trophozoite/schizont intraerythrocytic late stages, with no peak detected using the ABA-PA specific parameter conditions.
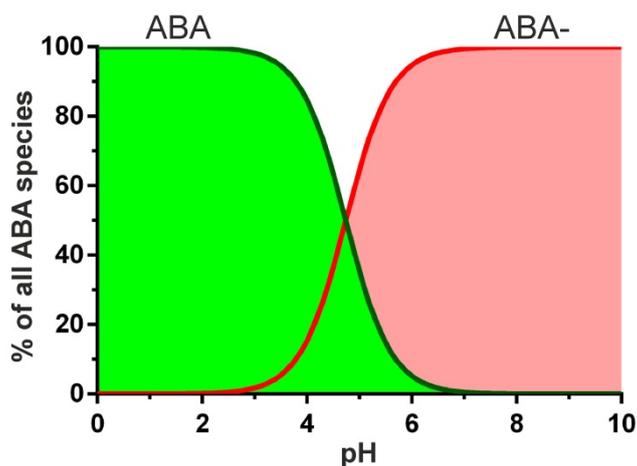
Fig. S6. ABA ionization species in solution as a function of pH.

Table S1. Recovery analysis of intra- and inter-day assays at high- and low-level ABA concentrations.

	Intra-day (n = 6)	Inter-day (n = 12)
Low level (0.15 ng ABA mL^{-1})	93±10%	96±10%
High level (1.5 ng ABA mL^{-1})	113±8%	116±9%

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