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Potential of Lichen Secondary Metabolites against *Plasmodium* Liver Stage Parasites with FAS-II as the Potential Target

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Abstract

Chemicals targeting the liver stage (LS) of the malaria parasite are useful for causal prophylaxis of malaria. In this study, four lichen metabolites, evernic acid (**1**), vulpic acid (**2**), psoromic acid (**3**) and (+)-usnic acid (**4**) were evaluated against LS parasites of *Plasmodium berghei*. Inhibition of *P. falciparum* blood stage (BS) parasites was also assessed to determine stage specificity. Compound **4** displayed the highest LS activity and stage specificity (LS IC₅₀ value 2.3 μM, BS IC₅₀ value 47.3 μM). The compounds **1-3** inhibited one or more enzymes (*Pf*FabI, *Pf*FabG and *Pf*FabZ) from the plasmodial fatty acid biosynthesis (FAS-II) pathway, a potential drug target for LS activity. To determine species specificity and to clarify the mechanism of reported antibacterial effects, **1-4** were also evaluated against FabI homologues and whole cells of various pathogens (*S. aureus*, *E. coli*, *M. tuberculosis*). Molecular modelling studies suggest that lichen acids act indirectly via binding to allosteric sites on the protein surface of the FAS-II enzymes. Potential toxicity of compounds was assessed in human hepatocyte and cancer cells (in vitro) as well as in a zebrafish model (in vivo). This study indicates the therapeutic and prophylactic potential of lichen metabolites as antibacterial and antiplasmodial agents.

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Notes

The authors declare no competing financial interest.

Supporting Information

The ¹H- and ¹³C-NMR spectra of compounds **1-4**, as well as primer and probe sequences used in qRT-PCR study. This material is available free of charge via the Internet at <http://pubs.acs.org>.

Malaria parasite *Plasmodium* has a complex life cycle, which includes two phases in the human host, liver stage (LS) and blood stage (BS). Malaria drug discovery has concentrated on targeting BS, not only because of direct clinical relevance, but also because the in vitro study of BS parasites is relatively cheap, easy and quick.¹ Although it is the first and an obligatory stage in the maturation and replication of parasites in the human host,² the LS has received far less attention due to low infection rates of liver cells and technical difficulties related to harvesting fresh parasites. Recently LS is being recognised as an essential target for malaria drug development and disease eradication for several reasons: i) for causal prophylaxis and prevention by halting the initiation of BS, ii) to prevent transmission in support of eradication efforts, iii) to reduce the risk of resistance development due to low parasite load, long residence time and single replication cycle, and iv) to target hypnozoites of *P. vivax* and *P. ovale* causing relapses.^{3,4} Primaquine, the only approved drug active against LS parasites and hypnozoites suffers from poor compliance, risk of haemolysis and high toxicity,⁵ hence new drugs are needed. Recent efforts have led to the discovery of synthetic compounds,⁶ and a few natural products of plant or microbial origin with LS inhibitory activity.^{7–11}

Several metabolic pathways, such as haem detoxification or nucleic acid metabolism, are involved in the BS action of antimalarial drugs. Many compounds with anti-LS activity inhibit similar metabolic pathways in BS parasites such as dihydrofolate reductase and cytochrome bc1 complex.⁶ Interestingly, the transcriptome and proteome expression levels of malaria parasites reveal that a large number of genes and proteins are expressed only in LS and hence represent stage-specific drug targets.^{12,13} The type II fatty acid biosynthesis pathway (FAS-II) has recently been shown to be crucial for survival of LS parasites but dispensable in BS parasites, thus appears to be the first target for solely prophylactic drugs.^{14,15} The FAS-II system involves a set of individual monofunctional enzymes, which is fundamentally different to the mammalian type I system (FAS-I) consisting of a dimer of a large multifunctional polypeptide. The sequence similarity between the enzymes of FAS-II and the corresponding domains of FAS-I are weak, although the individual steps in biosynthesis are essentially the same.¹⁶ A few synthetic compounds have been characterised with activity against LS and FAS-II enzymes; enoyl-ACP reductase (FabI) inhibitor triclosan,¹⁷ beta-ketoacyl-ACP reductase (FabG) inhibitor hexachlorophene,^{12,18} and 2-hexadecynoic acid (2-HDA), which inhibits three enzymes, FabI, FabG and FabZ (beta-hydroxyacyl-ACP dehydratase).¹⁹

Lichens are symbiotic associations between an exhabitant fungus and one or more inhabitant photosynthetic partners (algae or cyanobacteria). Numerous studies revealed a broad range of biological activities of lichen metabolites, including inhibition of gram-positive bacteria and mycobacteria.^{20,21} However, their mechanism of action has often remained unidentified. Lichens are traditionally used for a variety of purposes, e.g. as antibiotics, laxatives, antifebrile agents or against coughing (including that associated with tuberculosis).^{22,23} *Usnea* species are used for malaria and fever in Kenya²⁴ and the in vitro activity of (+)-usnic acid (**4**) against BS parasites has been confirmed.²⁵ Derivatives of **4** have recently been shown to inhibit LS parasites,²⁶ however to our knowledge, no study has yet reported the prophylactic potential of **4** or the other lichen compounds.

The aim of this study was to assess the malaria prophylactic and chemotherapeutic potential of four selected lichen metabolites, evernic, vulpic, psoromic and (+)-usnic acids (**1-4**) towards LS and BS parasites. To investigate the FAS-II enzymes as potential target for LS activity, inhibitory effects of the compounds were assessed against three *P. falciparum* FAS-II elongation enzymes, i.e. *PfFabI*, *PfFabG* and *PfFabZ*, prepared recombinantly in our laboratory. Cytotoxicity and hepatotoxicity of the compounds were determined in vitro [human cancer (KB) and human hepatocytes (HuH7.5) cell lines] and in vivo (zebrafish) models. The overall similarity between bacterial/mycobacterial and plasmodial FAS-II enzymes is significant,²⁷ and many known inhibitors show cross inhibition towards FAS-II enzymes of different origin.²⁸ Thus, to determine species-specificity and in an attempt to clarify the mechanism of the reported antibacterial and antimycobacterial effects, the lichen acids were also tested against FabI homologues from *M. tuberculosis* (*MtFabI*), *E. coli* (*EcFabI*) and *S. aureus* (*SaFabI*) and against the whole cell bacteria/mycobacteria. The molecules were docked to all six enzymes of interest to gain further information on their interactions with the enzymes.

RESULTS AND DISCUSSIONS

Antimalarial Activity and Plasmodial FAS-II Enzyme Studies

The malaria prophylactic potential of the lichen acids was tested against *P. berghei* LS parasites by assessment of infections after compound exposure with quantitative real time-PCR (qRT-PCR). All compounds showed activity with (+)-usnic acid (**4**) exhibiting the highest inhibitory effect with an IC₅₀ value of 2.3 μ M, followed by vulpic acid (**2**, IC₅₀ 10.2 μ M). Psoromic acid (**3**) had moderate LS activity (IC₅₀ 31.6 μ M), whereas evernic acid (**1**) showed low efficacy (IC₅₀ 77.3 μ M). To verify these results, the effect of the compounds on the morphology and development of LS parasites was assessed by immunofluorescence analysis (IFA) (Figure 1). In accordance with the qRT-PCR results, compounds **2** and **4** showed a decrease in the size of parasites at 10 μ M, whereas **1** and **3** had no effect at this concentration (Figure 1b). Further analysis of **4** demonstrated that a similar reduction in size could be achieved at a concentration of 1 μ M (Figure 1c). When tested against BS of multi-drug resistant *P. falciparum* K1 strain, **3** exhibited the best BS potential (IC₅₀ 29.2 μ M), which was comparable to its LS activity. Compounds **2** and **4** showed lower levels of BS activity (48.5 μ M and IC₅₀ 47.3, respectively) and were thus more specific against LS parasites. Particularly **4** had high selectivity for LS, with a 21-fold lower IC₅₀ value than that against BS. Evernic acid (**1**) had a marginal BS activity (IC₅₀ 142.1 μ M).

To investigate FAS-II enzymes as potential targets of the lichen compounds, the inhibitory activity of compounds **1-4** was evaluated against three key plasmodial FAS-II enzymes *PfFabI*, *PfFabG* and *PfFabZ* (Table 1). With the exception of **4**, all compounds exhibited moderate to very low activity towards at least one of the enzymes, with highest potential against *PfFabZ*. The most active compound was **1**, with the IC₅₀ values of 10.7 μ M and 36.1 μ M against *PfFabZ* and *PfFabI*, respectively. **3** was the only compound that inhibited all three enzymes, with least potency against *PfFabG*. **2** had considerable activity towards *PfFabZ* (IC₅₀ 20.5 μ M) only. This data however did not support clearly that FAS-II was the mechanism underlying the LS activity of the compounds. In order to shed more light on

FAS-II enzyme inhibition as biological target in LS parasites, detailed molecular docking studies were performed. Initially, the results of docking studies with the structural data of the *PfFabI*, *PfFabG* and *PfFabZ* enzymes by employing three different software packages (AutoDock, Vina and Glide) could not explain the experimentally obtained inhibition of the three enzymes. The interaction of ligands with the active sites of enzymes either could not be observed or the binding affinities were low. It is possible that due to the low flexibility of ligands, they could not fit into the enzyme active sites. Therefore, we have employed iGemDock to examine interaction of ligands using whole enzyme surfaces as targets. Docking was accompanied by control experiments using triclosan (an uncompetitive inhibitor of *PfFabI*),²⁹ hexachlorophene (competitive inhibitor of *PfFabG*),¹⁸ and epigallocatechin gallate (competitive inhibitor of *PfFabZ*)³⁰ as reference compounds. Interestingly, the ligands **1-4** do not bind at the enzyme active sites, but to cavities within the whole surface of the corresponding *PfFAS-II* enzymes (see Table 2 for binding energies), suggesting that the ligands bound to the surface may affect enzyme activity via different mechanisms. This effect is most prominent for *PfFabZ*, where the known standard, epigallocatechin gallate, blocks the entrance of substrate binding tunnel, an effect which has been observed also with other flavonoids.³¹ The lichen compounds **1-4** did not interact with the entrance of the substrate tunnel. (+)-Usnic acid (**4**) interacts furthest from the active site, thus does not affect activity, which is in accordance with the results obtained in enzyme assay (Figure 2).

Antibacterial/Antimycobacterial Activity and Target Determination Studies

Lichen metabolites are well known for their antibacterial and antimycobacterial effects, where FAS-II is a well-established target. In an attempt to clarify the mechanism of the reported antibacterial/antimycobacterial effects, **1-4** were tested for in vitro inhibitory potential against whole cells and FabI homologue enzymes from *M. tuberculosis* (*MtFabI*), *E. coli* (*EcFabI*) and *S. aureus* (*SaFabI*) (Table 1). In whole cells, **4** emerged as the most active compound against *M. tuberculosis* (MIC 15.1 μ M). The only other compound exhibiting some marginal antimycobacterial activity was **3** (MIC 122.9 μ M). None of the compounds arrested the growth of *E. coli* (IC₅₀ > 64 μ M), and **2** was the sole compound with moderate inhibitory effect against *S. aureus* (MIC 32 μ M).

The enzyme inhibition assays pointed out some selectivity in the inhibition of FabI enzyme from a range of microorganisms, but still failed to clearly identify FAS-II as their targets (Table 1). For example, **4**, which was inactive against all three plasmodial FAS-II enzymes, had marginal activity against *MtFabI* and *SaFabI*, and moderate activity against *EcFabI*. Compound **2**, which failed to inhibit plasmodial *PfFabI* and *MtFabI*, inhibited *SaFabI* and *EcFabI* with low potency. Also **1** showed low inhibition towards *SaFabI* and *EcFabI*. **3**, the sole compound interacting with all three *P. falciparum* elongation enzymes, was inactive against all bacterial/mycobacterial FabI analogues. Compounds **1-4** were docked to the analogue enzymes *MtFabI*, *EcFabI* and *SaFabI*. Although the binding affinities were better, the binding energies (Table 2) did not correlate well with the observed inhibition activities, thus the data is not clearly in support of *PfFabI* to be the drug target of **1-4**.

In vitro and In vivo Toxicity

Compounds **1-4** exhibited no in vitro cytotoxicity against the human hepatoma cell line HuH7.5 (IC₅₀ >100 μ M). When tested in vitro against the human cancer cell line KB, the obtained IC₅₀ values ranged between 31.6 μ M (**3**) to 190.4 μ M (**1**). In general in vivo toxicity studies towards zebrafish larvae at 4 days post-fertilisation (dpf), the maximum-tolerated concentrations (MTC) of **1-4** were determined to be 60.2 μ M, 15.5 μ M, 3.6 μ M, and 0.3 μ M, respectively. The compounds were then analysed for their hepatotoxic potential in *fabp10a:DsRed2* zebrafish larvae at concentrations up to and including their MTC. For **1-3**, liver phenotypes indicative of hepatotoxicity were seen in 40% of larvae at all concentrations tested. These phenotypes primarily included reduction or enlargement of liver size (Figure 3), both of which are indicative of drug-induced liver toxicity in zebrafish. For (+)-usnic acid (**4**), no hepatotoxicity was observed at the highest concentrations tested, up to and including its MTC (Figure 3). These initial results appear to suggest that **1-3** may have some intrinsic hepatotoxicity, which warrants further investigation. Compound **4**, while more potent overall with regard to general toxicity, did not exhibit any specific in vivo hepatotoxicity at the concentrations tested.

Significance of the Results

The inhibition of malaria LS parasites (by **1-4**), as well as BS parasites (by **1-3**) is being described for the first time. Semi-synthetic derivatives of **4** were recently shown to inhibit *P. yoelii* LS parasites,²⁶ but to our knowledge the LS activity of **4** itself has not been reported to date. In comparison to atovaquone, the standard used in this study because of its efficacy and low toxicity in cultured cells in vitro, the LS activity of **4** appears low. However, when compared to the reported in vitro LS activity of the only licensed liver schizonticide, primaquine (IC₅₀ 7.5 μ M in Huh7 and HepG2 cells in *P. berghei* model),^{2,6} **4** is almost four times more potent. The IC₅₀ value of primaquine towards BS of *Plasmodium* ranges between 1.4–3.3 μ M,^{32,33} making **4** more selective towards LS parasites. It has been reported that primaquine is extensively metabolised to become less active than some of its metabolites or derivatives.^{34,35} Usnic acid is known to metabolise to monohydroxylated and glucuronide conjugates in human liver fractions³⁶ but it remains uncertain whether **4** or its metabolite(s) eliminates the LS parasites. The reported in vitro BS activity of **4** against the K1 strain *P. falciparum*²⁵ is in accordance with our data. The lack of inhibitory activity of **4** against all PfFAS-II enzymes suggests that this pathway is not involved in its observed LS effect. Previous studies showed that the biosynthesis of vitamin E is of importance in LS parasites³⁷ and this pathway was recently shown to be inhibited by usnic acid in BS parasites.³⁸ However, whether the observed LS activity of **4** results from the inhibition of this pathway is speculative. On the other hand, hydrophobic compounds have a natural affinity for cell membranes and exert their antimicrobial effects generally by targeting the cell membrane.³⁹ Usnic acid, which is a weak acid with highly lipophilic properties has been reported to cause proton leakage in mitochondrial membranes and disrupt bacterial cell membranes.^{40,41} The effects of **4** (potentially the remaining lichen acids **1-3** as well) on malarial cells may involve a similar mechanism.

The observed antimicrobial activities of the lichen metabolites in this study are partly in agreement with previous reports. Reported antimycobacterial activity of (+)- usnic acid (**4**) ranges from similar to ten times lower potency.^{21,42,43} The low efficacy of **3** against *M. tuberculosis*⁴⁴ and *S. aureus*⁴⁵ is in agreement with the results obtained in this study. The antimycobacterial potential of **2** reported half a century ago⁴⁶ could not be confirmed in this work; and although several reports over the last 60 years described the antibacterial activity of **1**, **2** and **4** against *S. aureus*.^{20,47,48} **2** was the only compound inhibiting *S. aureus* with an IC₅₀ of 32 μ M, which is lower than that previously reported.⁴⁸ These discrepancies may stem from the use of different test systems and incompletely solubilized samples in biological assays. Lichen metabolites are well known for their poor solubility in water,⁴⁹ which has led to many drug formulation studies involving nanoencapsulation⁵⁰ and liposomes.⁵¹ Such highly lipophilic compounds may precipitate in the aqueous media and affect the outcome of the enzyme or whole cell-based bioassays. In this study, we ensured a complete solubility prior to any biological testing. The solubility problem experienced in DMSO at high test concentrations (>120 μ M) was overcome by brief agitation or sonication. The inactivity against *E. coli* confirms the poor potency of lichen secondary metabolites against gram-negative bacteria.⁵² Comparison of the whole cell activity with the enzyme activity suggests that *MtFabI* can not be related to the in vitro antimycobacterial effect of **4**.

Zebrafish (*Brachydanio rerio*) that share high genetic, physiological, and pharmacological similarities with humans are emerging as an attractive model for rapid in vivo hepatotoxicity analysis of drugs.^{53,54} Larval zebrafish have been used to test known hepatotoxic drugs and non-hepatotoxic compounds, with well-matched results.⁵⁵ (+)-Usnic acid (**4**) has been shown to be toxic for human hepatoblastoma HepG2 cells,⁵⁶ to cause liver damage in rats,⁵⁷ and associated with liver toxicity in humans.⁵⁸ However in the current study, which used a luciferase-based cell viability assay, it did not show in vitro cytotoxicity against the human hepatoma cell line HuH7.5 at concentrations up to 100 μ M. Notably, **4** did not exhibit any specific in vivo hepatotoxicity towards zebrafish larvae even at the highest test concentrations. Nevertheless, given the relatively low MTC of **4** in zebrafish larvae – thereby causing any potential hepatotoxicity to be masked by general toxicity – further in vivo investigations of **4** with respect to compound-induced liver injury are warranted. The other three compounds did not exhibit in vitro toxicity in HuH7.5 cells, but appeared to have potential dose-dependent hepatotoxicity in vivo (zebrafish).

CONCLUSIONS

Current malaria drug discovery and development efforts focus on the well-established blood stage (BS), by using both phenotypic and target-based approaches. Drugs targeting the LS parasites can be used for causal malaria prophylaxis with low risk of resistance due to low parasite load in the hepatic stage. Hence, LS could present additional options for the control of malaria when a truly high throughput screening system is established and novel targets are developed. The present study identified (+)-usnic acid (**4**) as the most active and the most selective LS inhibitor. Molecular modelling studies with FAS-II enzymes as drug target suggest the mechanisms of action of lichen acids to be different to those of known ligands. They appear to act indirectly via binding to allosteric sites on the protein surface,

which possibly affects the enzyme conformation and subsequently interferes with enzyme activity. Biological targets underlying antimalarial, antimycobacterial and antibacterial activities of **1-4** warrant further studies. This study also highlights the need to further explore the in vivo hepatotoxic and cytotoxic potential of lichen acids. Future optimisation studies by medicinal chemistry and drug formulation could improve the antimicrobial/antimalarial activity and the solubility while substantially reducing the hepatotoxicity of lichen acids.

EXPERIMENTAL SECTION

Materials

Evernic, vulpic and psoromic acids (**1-3**) were purchased from Extrasynthese (France), whereas (+)-usnic acid (**4**) was purchased from Aldrich. Their purity and identity were confirmed by their 1D NMR data, which was in good agreement with those reported in the literature.⁵⁹⁻⁶² NMR spectra were obtained using Jeol 400 MHz and Agilent 600 MHz spectrometers in CDCl₃ or DMSO-*d*₆.

In vitro Antiplasmodial BS Assay

Chloroquine and pyrimethamine resistant *P. falciparum* K1 strain in vitro culturing method and ³H-hypoxanthine incorporation assay were carried out in duplicates as previously described,⁶³ with the modifications of 15 μM hypoxanthine (Sigma) in assay medium, 0.4% parasitemia and 48 h incubation of the compounds before addition of ³H-hypoxanthine.

In vitro Antiplasmodial LS Assay

Liver stages of *P. berghei* ANKA in the hepatoma cell line HuH7.5 were used and sporozoites were freshly dissected from infected *Anopheles* mosquitoes (*PbGFP*) (shipped from the New York University Insectary Core facility). HuH7.5 cells were seeded in 48-well plates (60,000 cells/well) and infected with 30,000 *PbGFP* sporozoites. Infected cells were allowed to develop for 48 h in the presence of the compounds at 37 °C in a CO₂ incubator with media changes every 24 h. Total RNA was extracted from infected cells using TRIzol (Life Technologies) according to the manufacturer's instructions. Samples were subsequently treated with TURBO DNA-free DNase (Ambion) to remove any contaminating genomic DNA. First strand cDNA was synthesised from 2500 ng of total RNA using the Maxima First Strand cDNA Synthesis Kit (Fermentas/Thermo Scientific). The reaction was then diluted 4-fold with water and used as the cDNA template for individual PCR reactions. The assessment of *Plasmodium* infection was done using qRT-PCR. Plasmid controls were constructed by amplifying the human RPS11 and *P. berghei* 18S rRNA (*Pb18S*) target using forward and reverse primers (Integrated DNA Technologies, Inc), and cloning the gene fragments into pGEM-T (Promega) by standard protocols. The plasmids were used for DNA based standard curves to interpolate absolute numbers of the gene targets in the DNA sample of either the RPS11 or *Pb18S* targets. For qRT-PCR, 0.5 μL of the diluted cDNA was used in the 10 μL reaction including FastStart Universal Probe Master (Roche) with 0.5 μM gene-specific forward and reverse primers and probes. qRT-PCR reactions were performed in a StepOnePlus Real-Time PCR system (Applied Biosystems) with the following cycling conditions: 50 °C 2 min, 95 °C 10 min, 50

cycles of 95 °C 15 s, 60 °C 1 min. The data was analysed using the StepOne Software v2.2.1 (Applied Biosystems). The normalised reduction in *PB18S* gene expression from infections treated with the compounds compared with DMSO treated controls was calculated and used for IC₅₀ estimation using the ICEstimator program (<http://www.antimalarial-icestimator.net/>). Compounds were tested at concentrations of 10 µM to 100 µM in complete media and atovaquone was used as a positive control. To verify the results of the assay and to determine the effect of the compounds on the LS morphology and development, immunofluorescence analysis (IFA) of *P. berghei*-infected HuH7.5 cells after compound treatment was carried out. For IFA analyses, 8-well chambered slides (Millipore) were seeded with HuH7.5 (50,000/well) cells and infected with *P. berghei* GFP sporozoites (33,000/well) as indicated previously. After 48 h of incubation, the infected cells were fixed with 10% neutral buffered formalin. IFA was carried out using a mouse monoclonal antibody against the *Plasmodium* HSP70 protein. The slides were scanned with a fluorescence microscope (Olympus Provis III) and images were captured using a 40X objective.

Antibacterial Assays

Whole cell experiments against H₃₇R_v strain of *M. tuberculosis* (ATCC-27294),⁶⁴ *S. aureus* (ATCC-6538)⁶⁵ and *E. coli* (ATCC-8739)⁶⁵ were carried out (in triplicates) as reported.

In vitro Toxicity

The cytotoxicity of compounds was assessed against KB cells (human carcinoma cells of the nasopharynx) as previously described.⁶⁶ In addition, toxicity was assayed against human hepatocytes using the Cell-titerGlo Luminescence assay (Promega). The viability of HuH7.5 cells incubated with compounds for 24 h was compared with cells incubated with 0.1% DMSO according to manufacturer's instructions. HuH7.5 cells were seeded in 96-well opaque-walled plates (30,000/well). The cells were grown in complete media (advanced DMEM supplemented with 10 FBS, 2% penicillin/streptomycin, 2% glutamine, non-essential amino acids, and 1% amphotericin) to which 0.1% DMSO or various concentrations of compounds were added. After 24 h, 100 µL of cell titer-Glo reagent was added and luminescence was measured using the Veritas luminometer (Promega).

In vivo Hepatotoxicity

To determine the hepatotoxic effects of lichen acids, a transgenic line with liver-specific expression of DsRed was used (*fabp10a:DsRed2*). Zebrafish embryos were raised in incubators at 28.5 °C with a 14 h light/10 h dark cycle. Larval zebrafish at 3 day post-fertilisation (dpf) were sorted for liver fluorescence and treated with compounds in 24-well plates, with 10 larvae per well. At 4 dpf, larvae were checked for general toxicity and heart toxicity. Compounds were screened for hepatotoxicity up to the maximum-tolerated concentration (with regard to general toxicity). At 6 dpf, liver toxicities were scored by fluorescence microscopy (Leica MZ10F). Five liver phenotypes were scored: normal liver, reduced fluorescence, reduced size, enlarged liver and absent liver.

Enzyme Assays

Plasmodial FAS-II enzyme inhibitory assays were carried out (in duplicates) as previously described with minor modifications.³⁰ Briefly, all measurements were performed in 20 mM HEPES, pH 7.4, and 150 mM NaCl in a total volume of 1 mL and *Pf*FabZ activity was measured at 263 nm for 2 min in the presence of 25 μ M crotonoyl-CoA and 1 μ g of enzyme. Reference compounds were triclosan (*Pf*FabI) and (–)-epigallocatechin gallate (*Pf*FabG, *Pf*FabZ). *Mt*FabI (50 nM) and *Ec*FabI (10 nM) were assayed in 30 mM PIPES and 150 mM NaCl buffer at pH 6.8 or pH 8.0, respectively, using 25 μ M 2-dodecenoyl-CoA and 250 μ M NADH while *Sa*FabI (50 nM) was assayed in 100 mM HEPES buffer at pH 7.8 in the presence of 10 μ M dodecenoyl *Sa*ACP and 250 μ M NADPH. Reference compound for all three enzymes was triclosan. Enzyme activity was monitored by following the oxidation of NADH or NADPH at 340 nm. The initial velocities were determined in triplicate at increasing inhibitor concentrations [I], and IC₅₀ values were calculated by fitting the data to equation $y = 100/(1 + [I]/IC_{50})$ using Grafit 4.0.

Molecular Modelling

The initial 3D structures of studied compounds were obtained using ChemBioOffice 12 (PerkinElmer Informatics, 2012) and processed using LigPrep (LigPrep, version 2.5, Schrödinger, LLC, New York, NY, 2011) to generate ligand conformations and protonation states of their ionisable groups at pH 7.4. Molecular properties were calculated using VegaZZ^{67,68} and QikProp software (QikProp, version 3.4, Schrödinger, LLC, New York, NY, 2011). The coordinates of proteins *Pf*FabZ, *Pf*FabG, *Pf*FabI, *Sa*FabI, *Ec*FabI and *Mt*FabI targets for molecular docking (PDB entries 1z6b, 2c07, 1v35, 3gr6, 1c14 and 1p45, respectively), were imported into the Maestro (Maestro, version 9.2, Schrödinger, LLC, New York, NY, 2011) and processed using “Protein Preparation Wizard” workflow to remove water molecules, set atom types, add hydrogen atoms for proteins at pH 7.4 and resolve the problems with protein structures. Molecular docking was carried out using iGemDock⁶⁹ software using default settings and the whole surfaces of proteins were used as target.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Immunofluorescence microscopy visualising *P. berghei* parasites 48 h post-infection. Images shown depict liver stage parasites detected by an antibody against parasite protein HSP70 (FITC, green) and stained with Hoechst nuclear stain (blue) at 40X objective magnification. a) Infected cultures grown in the presence of positive control Atovaquone (Ato) at three separate concentrations and 0.1% DMSO control. b) Infected cultures grown in the presence of four lichen metabolites at the concentration of 10 μ M. c) Infected cultures grown in the presence of three separate concentrations of (+)-usnic acid (**4**) and a 0.1% DMSO control.

Figure 2.

The solvent accessible surface representation of *PfFabZ* and location of most favourable binding sites of lichen acids **1** (red), **2** (blue), **3** (green) and **4** (cyan). The protein is shown in a) sideways, b) top and c) bottom orientation. Ligands are shown in CPK representation and the arrow indicates the position of the protein active site. **1** is buried in the protein surface, while **4** is farthest from the protein active site.

Figure 3.

Hepatotoxic effects of lichen acids **1-4** in zebrafish larvae. *fabp10a*:DsRed2 larvae are shown at 6 dpf after 3 days of exposure to compounds. a) control larvae treated with 1% DMSO only; b) larva treated with 60.2 μ M of **1**, revealing reduced liver size; c) larva treated with 15.5 μ M of **2**, revealing enlarged liver; d) larva treated with 0.3 μ M of **4**, revealing liver with normal shape and size.

Table 1

IC₅₀ Values (μM) of Lichen Metabolites 1-4 Against Various FAS-II Enzymes

compounds	<i>PfFabI</i>	<i>PfFabG</i>	<i>PfFabZ</i>	<i>MtFabI</i>	<i>SaFabI</i>	<i>EcFabI</i>
control	0.05 ^a	1.1 ^b	0.7 ^b	1.0 ^a	0.025 ^a	0.005 ^a
evermic acid (1)	36.1	>200	10.7	>200	170	90.0
vulpic acid (2)	>140	>200	20.5	>200	90.0	84.0
psoromic acid (3)	71.4	183	35.2	>200	>200	>200
(+)-usnic acid (4)	>200	>200	>200	190	190	47.0

Reference compounds ^atriclosan, ^bepigallocatechingallate

Table 2

Binding Energies (kcal/mol) of Lichen Metabolites 1-4 to Protein Targets

compound	<i>PjFabZ</i>	<i>PjFabG</i>	<i>PjFabI</i>	<i>MtFabI</i>	<i>SaFabI</i>	<i>EcFabI</i>
control	-113.9 ^a	-90.3 ^b	-84.5 ^c	-83.2 ^c	-80.1 ^c	-88.0 ^c
evermic acid (1)	-101.7	-114.3	-121.0	-101.1	-109.1	-111.1
vulpic acid (2)	-93.0	-103.9	-96.2	-100.4	-97.1	-96.2
psoromic acid (3)	-92.9	-117.3	-100.1	-117.4	-100	-99.7
(+)-usnic acid (4)	-88.7	-121.7	-106.9	-100.7	-117.1	-106.4

Control ligands ^aepigallocatechin gallate, ^bhexachlorophene, ^ctriclosan