The A/T-specific DNA alkylating agent adozelesin inhibits Plasmodium falciparum growth in vitro and protects mice against Plasmodium chabaudi adami infection

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Abstract

There is an urgent need for new anti-malarial drugs to combat the resurgence of resistance to current therapies. To exploit the A/T richness of malaria DNA as a potential target for anti-malarial drugs we tested an A/T-specific DNA synthesis inhibitor, adozelesin, for activity against Plasmodium falciparum in vitro and Plasmodium chabaudi adami in mice. Adozelesin is a DNA alkylating agent that exhibits specificity for the motif A/T, A/T and A. In P. falciparum 3D7 cultures, adozelesin acts as a powerful inhibitor of parasite growth (IC50 of 70 pM) and is equally potent at killing the drug-resistant strains FCR3 and 7G8. Using a real-time PCR assay, we show that treatment with adozelesin in vitro results in damage of P. falciparum genomic DNA. In synchronized cultures, adozelesin exhibits a concentration-dependent effect on parasitemia and on the development of parasites through the asexual cycle. In asynchronous cultures, parasites arrest at all stages of the asexual cycle suggesting that adozelesin exerts other anti-parasitic effects in addition to inhibiting DNA replication. These anti-parasite effects are irreversible since cultures exposed to adozelesin for more than 6 h fail to recover upon removal of the drug. Furthermore, adozelesin is very effective at suppressing malaria infection in vivo; growth of P. c. adami DK in mice was highly impaired by a single injection of adozelesin (25 μg/kg) at 4 days post-infection. These results demonstrate that adozelesin irreversibly blocks parasite growth in vitro and suppresses parasite infection in vivo, suggesting that A/T-specific DNA damaging agents represent a new class of compounds with potential as anti-malarials.

Keywords: Plasmodium falciparum; Plasmodium chabaudi adami; Malaria; Adozelesin; DNA damage; Chemotherapy

1. Introduction

Malaria is a devastating parasitic disease that kills more than one million people every year [1,2]. This disease primarily affects pregnant women and children in the developing world. While chemotherapy exists, resistance to this therapy is widespread and there is decreasing availability of drugs to treat this disease [3]. The recent completion of the genome sequence of Plasmodium falciparum, the most lethal causative agent of human malaria, offers new opportunities to identify more effective targets for drug development and to gain insight into the biological pathways involved in malaria pathogenesis [4].

P. falciparum DNA is disproportionately rich in adenine (A) and thymine (T) base pairs (80% A/T) compared to approximately 40% A/T in human DNA. One potential strategy to develop new anti-malarial drugs is to exploit this difference in genomic composition using A/T-specific DNA-binding drugs. These minor groove-binding compounds are currently under investigation as treatments for human cancers. Bioinformatic analyses have revealed that several of these compounds exhibit a strong preference for regions of genomic DNA that contain repeated A/T sequences [5]. These sequences are often prevalent within regions of mammalian genomic DNA that anchor the DNA to the nuclear matrix, termed matrix attachment regions (MAR). MAR loci are associated with sites of active DNA replication and transcription within the nucleus [6]. Consistent with this, treatment of human and rodent cell lines with A/T-specific
DNA-binding compounds results in lethal defects in DNA replication [7].

In *P. falciparum*, DNA replication occurs at multiple stages throughout the life cycle of the parasite, at times with exceptional efficiency. Inhibitors of DNA replication therefore hold the potential to block parasite growth at both the liver and blood stages of infection in humans. A number of DNA replication inhibitors have been tested against *P. falciparum* in vitro and not surprisingly, the most effective were those specific to A/T-rich sequences [8–10]. One compound in particular, CC-1065, exhibited very potent anti-malarial activity in vitro with an IC50 in the picomolar range [9]. However, a delayed death phenotype observed in mice following treatment with this compound precluded further testing in vivo [11].

Adozelesin is a synthetic analogue of CC-1065 that has been shown not to cause delayed death in mice [12,13]. Atozolezis is a member of the cyclopropylpyrroloindole (CPI) family, and shares the same potent anti-tumor activity in vitro and sequence specificity for the motif 5′-(A/T) (A/T) A-3′ as its parent CC-1065 compound [14,15]. Given the density of this motif within the *P. falciparum* genome, we tested whether adozelesin exhibits anti-malarial activity. In this study, we show that *P. falciparum* parasites are extremely sensitive to adozelesin in vitro. Parasites display a terminal phenotype that is not specific to defective DNA replication, suggesting that drug adducts formed on parasite DNA interfere with additional processes that are critical to parasite growth. We also show that adozelesin is efficient at suppressing parasite infection in a murine model of malaria. These results demonstrate that malaria DNA is highly susceptible to DNA damage by A/T-specific agents and suggest that this mode of action potentially offers an effective strategy against malaria.

2. Materials and methods

2.1. Parasite strains

In vitro studies were carried out using *P. falciparum* strains 3D7, FCR3 and 7G8. The 3D7 strain was obtained from MR4 (MRA-102, ATCC, Manassas, VA, USA), and both the FCR3/A2 and 7G8 strains were a kind gift from Dr. E. Georges (McGill University). The drug sensitivities of the three strains to chloroquine and pyrimethamine were verified to confirm the resistance status of these strains. The murine malaria *Plasmodium chabaudi adami* DK strain (isolate 556KA) was used for the in vivo experiments. This strain was kindly provided by Dr. D. Walliker (University of Edinburgh).

2.2. Parasite culture

*P. falciparum* parasites were cultured in human erythrocytes at 3–5% hematocrit in complete media (RPMI medium supplemented with 25 mM Hepes, pH 7.5, 0.225% sodium bicarbonate, 40 μg/ml gentamicin sulphate, 11 mM glucose, 200 μM hypoxanthine and 0.5% Albumax II (Invitrogen)) essentially as described [16]. Cultures were maintained in a modular chamber (Billups-Rothenberg) at 37 °C in a gas mixture of 1% O2 and 5% CO2. Cultures were synchronized at the ring stage by sorbitol lysis [17]. Parasitemia was determined by counting the number of infected red blood cells per 2000 red blood cells from Giemsa-stained thin smears.

2.3. Compounds

Adozelesin (U-73975) was a generous gift from Pfizer. A stock solution (2 mg/ml) was prepared in dimethylacetamide. For in vitro studies, adozelesin was further diluted in DMSO and stored at −20 °C in the dark. For in vivo studies, adozelesin was diluted in a PET/glucose solution (1.6 ml of polyethylene glycol 400, absolute ethanol and Tween 80 in a 6:3:1 ratio, mixed with 3.2 ml of 5% glucose) with a final concentration of 2% DMSO. Chloroquine (Sigma) was prepared as a stock solution (0.1 M) in water and stored at −20 °C. Pyrimethamine (Sigma) was prepared as a stock solution (10 mg/ml) in 1% acetic acid and freshly diluted in sterile water prior to use. Apicidinol (Sigma) was prepared as a stock solution (5 mM) in DMSO and used at a final concentration of 5 μM in cultures.

2.4. In vitro growth inhibition assay

In vitro growth assays were performed essentially as described [18]. *P. falciparum* cultures were diluted to 1% parasitaemia and 2% hematocrit in culture medium containing low concentrations of hypoxanthine (10 μM). Atozolezis or DMSO was added and 0.2 ml cultures were plated in triplicate in 96-well flat-bottomed plates alongside uninfected erythrocytes as controls. Plates were incubated at 37 °C for 24 h followed by the addition of 1 μCi of [1H(G)]-hypoxanthine (Amersham) to each well. The plates were incubated for a further 24 h, then harvested onto glass fiber filter mats using a cell harvester (Packard). The background cpn values from the uninfected samples were subtracted from the experimental values. The incorporation of 1H-hypoxanthine for drug-treated samples is expressed as the percent uptake relative to the DMSO control. IC50 values were calculated using a non-linear regression sigmoidal dose-response analysis (Prism v. 4.0a).

2.5. Extraction of genomic DNA

Parasites were released from red blood cells upon treatment with 0.01% saponin in RPMI for 10 min on ice. Free parasites were recovered after centrifugation at 5000 × g for 5 min, and washed several times with cold PBS. *P. falciparum* genomic DNA was isolated as described previously [19]. Parasite pellets were resuspended in 0.1 ml of lysis buffer (400 mM Tris-HCl, pH 8.0, 60 mM EDTA, 150 mM NaCl and 1% SDS) and 1.5 M sodium perchlorate. Samples were incubated for 20 min at 37 °C and then 20 min at 65 °C. Nucleic acids were recovered by chloroform extraction and precipitated with ethanol. The DNA pellet was dissolved in 20 μl of water. DNA concentrations were verified using spectrophotometric analysis.
2.6. In vitro treatment with adozelesin

The pDONR entry vector (Gateway, Invitrogen) containing the \textit{P. falciparum} H103 gene (PF10_00352) cloned into the attB sites was used to analyze the effects of adozelesin on plasmid DNA in vitro (gift from C. Santamaria, McGill University). Reactions were carried out as described [14] and consisted of 1 µg of plasmid or genomic DNA, adozelesin (20 µM) or DMSO, in a 20 µl volume containing 0.1 × SSPE. Reactions were incubated at room temperature overnight. Plasmid DNA was purified using a PCR clean-up kit (Qiagen). Genomic DNA was precipitated with 0.3 M sodium acetate and ethanol and resuspended in 20 µl of TE.

2.7. Real-time PCR assay

Real-time PCR was used to monitor DNA damage induced by adozelesin treatment in vitro. We used a method previously established to monitor DNA damage in cell lines in response to other mutagenic agents in vitro [19]. Equal concentrations of the template DNA were verified by agarose gel electrophoresis following in vitro drug treatment of 1 µg of plasmid or genomic DNA (Section 2.6). PCR amplification reactions consisted of 12.5 µl Quantitect 2X Sybr Green Reaction Mix (Qiagen), 0.2 µM of each oligonucleotide and 1 µl of plasmid or genomic DNA in a 25 µl reaction volume. Real-time PCR was carried out in a Rotorgene thermocycler with the following cycling conditions: 95°C for 15 min, followed by 40 cycles of 95°C for 45 s, 50°C for 30 s and 60°C for 60 s. Oligonucleotides used to amplify the H103 gene were: 5′-ATT GAA GTA TGG AGG GTT TG-3′ (forward primer) and 5′-ATT TAC ATT ATC TTC TCT-3′ (reverse primer).

2.8. Efficacy of adozelesin against \textit{P. c. adami} infection in mice

To determine the effects of adozelesin on infections with the murine malaria \textit{P. c. adami} DK, groups of four female BALB/c mice (average weight 18–20 g) were injected by i.p. with 5 × 10^5 infected erythrocytes from an infected donor mouse [20]. At 4 days post-infection, each group of mice was injected i.p. either with 25 µg/kg of adozelesin or the PET/glucose/DMSO solution alone. Blood films from tail blood were prepared from each animal until day 13 p.i. Slides were fixed in methanol, stained with Giemsa and examined microscopically to determine the parasitemia. At least 500 erythrocytes were counted per slide using a ‘blind count’ method.

3. Results

3.1. Dose response of \textit{P. falciparum} cultures to adozelesin

To determine the anti-malarial effects of adozelesin, in vitro cultures of the 3D7 parasite strain were treated with increasing concentrations of the compound for 48 h. Both asynchronous and ring-stage synchronized cultures exhibited the same concentration-response with a 50% inhibitory concentration of 70 pM (Fig. 1A). The IC50 for adozelesin is substantially lower than that for chloroquine in the 3D7 strain under our conditions (20 nM, data not shown).

In addition to the 3D7 strain, the concentration-response was examined for two drug-resistant parasite strains. FCR3 is resistant to chloroquine while 7G8 is resistant to both chloroquine and pyrimethamine. The sensitivities of both strains to these anti-malarials was verified and agreed with published IC50 values (data not shown). Treatment of these drug-resistant strains resulted in the same concentration-response curves observed with 3D7 (Fig. 1B). This suggests that the mode of action for adozelesin is independent of the mechanism of 4-aminoquinolines and dihydrofolate reductase inhibitors.

3.2. Adozelesin damages \textit{P. falciparum} DNA in vitro

It is well established that adozelesin exerts its effects in human and rodent cell lines through the alkylation of adenine residues in genomic DNA [7]. The drug-DNA adducts that are formed are thought to cause the stalling of replication forks.
during DNA replication, leading to checkpoint activation and subsequent apoptosis [7,21–23]. To determine whether adozelesin induces DNA damage on \( P. falciparum \) DNA, we used a real-time PCR assay. In this assay, the amount of PCR product amplified is inversely proportional to the extent of DNA damage in the template DNA [19]. To validate this technique for treatment of DNA with adozelesin, we treated plasmid DNA with 20 \( \mu \)M adozelesin or DMSO for 18 h in vitro, purified and used as the template for real-time PCR with H103 oligonucleotides. Filled triangles represent the PCR amplification curves for DMSO-treated samples performed in triplicate and filled circles represent the curves for the adozelesin-treated DNA performed in triplicate. Experiments were repeated three times.

We next tested whether genomic DNA from \( P. falciparum \) cultures would also be susceptible to DNA damage detected in this assay. Genomic DNA was isolated from asynchronous 3D7 cultures and treated with 20 \( \mu \)M adozelesin or DMSO and used this treated DNA as the template in the PCR assay. This plasmid contains the \( P. falciparum \) H103 gene within the attB sites of the pDONOR vector and oligonucleotides were used to amplify the H103 gene for the PCR reaction. As shown in Fig. 2A, treatment of plasmid DNA with adozelesin resulted in a significant reduction of PCR product relative to the DMSO-treated control. Comparison of the cycle threshold (Ct) values from the PCR reactions suggests that there is at least 50-fold less H103 gene product amplified in the drug-treated samples.

We next tested whether genomic DNA from \( P. falciparum \) cultures would also be susceptible to DNA damage detected in this assay. Genomic DNA was isolated from asynchronous 3D7 cultures and treated with 20 \( \mu \)M adozelesin or DMSO in vitro. The genomic DNA was precipitated and served as the template in the real-time PCR assay to amplify the H103 gene. Similar to the plasmid DNA, we observed a decrease in the amplification of H103 from the adozelesin-treated template compared to the control (Fig. 2B). In this case, there was approximately a 30-fold reduction in the amount of PCR product following drug treatment relative to the control. These results suggest that adozelesin is capable of inducing damage to \( P. falciparum \) DNA, either in the context of a recombinant DNA sequence or in genomic DNA isolated from parasite cultures.

3.3. Adozelesin inhibits parasite development during asexual stages

Based on the concentration-response experiments from Fig. 1A, adozelesin treatment can effectively block the incorporation of radio labelled hypoxanthine in the standard radiometric assay for parasite growth. This assay measures the incorporation of hypoxanthine into both DNA and RNA during DNA replication and transcription. To determine the phenotypic effects of this inhibition on parasite growth and development, we monitored the parasitemia of synchronized asexual cultures after continuous adozelesin treatment during a single 48 h cycle (Table 1). Treatment with concentrations of adozelesin of 0.2 nM or greater resulted in a significant reduction in parasitemia relative to the control.

In the blood stage of \( P. falciparum \), DNA replication occurs in the late trophozoite to early schizont stage. Treatment of synchronized cultures with aphidicolin, an inhibitor of DNA polymerase alpha activity in \( P. falciparum \) [25] and many other organisms, blocks the parasitemia of the cultures and arrests parasites at the late trophozoite/early schizont stage (Table 1). When cultures were treated with adozelesin, we observed a concentration-dependent effect on parasite development (Table 1). Parasites exposed to DMSO or 20 \( \mu \)M adozelesin progressed from early rings through the first asexual cycle and formed new rings in the second cycle. At a 0.2 nM concentration, parasites arrested primarily at the trophozoite stage of the first cycle, similar to the effect with aphidicolin. This is consistent with adozelesin inhibiting some aspect of DNA replication in the parasites. Surprisingly, treatment with higher concentrations of adozelesin arrested parasites at earlier stages of the asexual cycle. Cultures treated with 2 and 20 nM adozelesin predominantly consisted of early ring-stage parasites. These rings arrested within the first asexual cycle as confirmed by
microscopic examination of parasites after 24 h of drug treatment (data not shown). These observations, taken together with the inhibition of parasitemia in the cultures at concentrations of 0.2 nM or greater, imply that adozelesin exhibits a concentration-dependent block of parasite development throughout the asexual stages.

The results presented above suggest that adozelesin does not selectively inhibit DNA replication in the parasite since, at drug levels above 0.2 nM, the parasite growth is arrested at the ring stage before DNA replication begins. One possibility is that this compound has a specific effect on the transition from the ring stage to the trophozoite that causes the early ring arrest at higher doses. To test this, asynchronous cultures were treated with different concentrations of adozelesin for 48 h and the distribution of parasites at different developmental stages was evaluated by counting the percent rings in the starting culture and also following the percentage of parasites at different developmental stages was evaluated by microscopic examination of parasites after 24 h of drug treatment (data not shown). Asynchronous cultures were treated with DMSO or increasing concentrations of adozelesin for 24 h before washing out the drug. To address this, cultures were treated with 2 nM adozelesin for 24 h before washing out the drug. Data represent the average of three experiments performed in triplicate ± S.E.M. Aphidicolin served as a positive control. The results presented above suggest that with lower concentrations of adozelesin, there is insufficient accumulation or activation of the compound in the parasite nucleus within the 24 h period of drug treatment to exert its full cytotoxic effects. One possible explanation could be the slow penetration of the drug into the parasite causing a temporal delay in drug accumulation.

To address this, cultures were treated with 2 nM adozelesin for varying times and the ability to resume growth was determined by microscopic examination of parasites after 24 h of drug treatment (Fig. 3A).

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The susceptibility of P. falciparum parasites to adozelesin in vitro prompted us to test whether this compound would be...
in one mouse in this second trial was due to a treatment failure. The four mice. It is possible that the lack of response to adozelesin treated group, malaria infection was suppressed in three out of resolved the infection by day 13 p.i. (Fig. 4B). In the adozelesin- a peak parasitemia of 7.6–9.4% at day 9 p.i. and most mice (Fig. 4A). In the second trial, all of the control mice reached pressed throughout the duration of the trial in all four animals parasitemia in the adozelesin-treated mice was effectively sup- mal displayed a delay in the course of infection. In contrast, the infection in control mice progressed to a peak parasitemia of 6.9–12% before beginning to resolve (Fig. 4A). One control ani- et B). In both trials, early parasite infection was detectable this dose of adozelesin[12]. Parasitemia was monitored in these animals. Filled circles represent the parasitemia from the adozelesin-treated animals. (B) Repeat of animal trial described in (A) with parasitemia monitored from day 4 p.i. to day 13 p.i.

Fig. 4. In vivo anti-malarial activity of adozelesin in mice. (A) Two groups of four female BALB/c mice were infected with $5 \times 10^5$ parasites from the strain P. c. adami DK. Four days p.i., one group was injected i.p. with a single dose of adozelesin (25 g/kg) while the other group received the vehicle alone. Parasitemia was monitored from 3 to 10 days p.i. from Giemsa-stained blood films of tail blood. Filled triangles represent the parasitemia from the control animals. Filled circles represent the parasitemia from the adozelesin-treated animals. (B) Repeat of animal trial described in (A) with parasitemia monitored from day 4 p.i. to day 13 p.i.

effective as an anti-malarial in vivo. This was tested using the avirulent parasite strain P. c. adami DK as a model for murine malaria. Mice were infected with $5 \times 10^5$ parasites, followed by a single dose i.p. injection of adozelesin (25 g/kg). 4 days post-infection (p.i.). No toxicity has been reported in mice for this dose of adozelesin[12]. Parasitemia was monitored in these mice beginning on day 3 or 4 p.i. to days 10–13 p.i. (Fig. 4A and B). In both trials, early parasite infection was detectable at 4–5 days p.i. prior to drug injection. In the first trial, the infection in control mice progressed to a peak parasitemia of 6.9–12% before beginning to resolve (Fig. 4A). One control ani- mal displayed a delay in the course of infection. In contrast, the parasitemia in the adozelesin-treated mice was effectively sup- pressed throughout the duration of the trial in all four animals (Fig. 4A). In the second trial, all of the control mice reached a peak parasitemia of 7.6–9.4% at day 9 p.i. and most mice resolved the infection by day 13 p.i. (Fig. 4B). In the adozelesin- treated group, malaria infection was suppressed in three out of the four mice. It is possible that the lack of response to adozelesin in one mouse in this second trial was due to a treatment failure. We suggest that adozelesin did not reach the peritoneum in that particular animal resulting from an error during the drug admin- istration. These results suggest that adozelesin exhibits potent anti-malarial effects in vivo and that A/T-specific DNA-binding compounds could constitute a new class of anti-malarial drugs.

4. Discussion

There is an urgent need to expand the arsenal of anti-malarial compounds in order to combat this major public health prob- lem. The widespread prevalence of parasites resistant to current therapies suggests that new compounds are needed that target alternative parasite substrates. One potential target is parasite DNA that could be achieved by exploiting the disproportional A/T richness of malaria DNA with compounds that bind specific- ally to A/T motifs. It has been observed that compounds with this sequence selectivity have the lowest known IC50 values in cultures of P. falciparum [9], however, little evidence supports their efficacy against malaria in vivo.

In this study, we have characterized the anti-malarial effects of one of these compounds, adozelesin, both in vitro and in vivo. We have shown that the IC50 values for adozelesin against drug-sensitive and drug-resistant strains of P. falciparum are extremely low, in the picomolar range and the lethal effects are exerted within the first 48h of the asexual cycle in vitro. This effect is irreversible, yet dependent on a minimum time of exposure to the drug. This differs from mammalian cells in which cytotoxic effects of adozelesin are observed following 2 h of treatment at picomolar concentrations [7,15]. One important issue affecting the adozelesin efficacy may be the accessibility of the parasite to the compound. During the intraerythrocytic cycle, the parasites are contained within a parasitophorous vacuole nested within the cytoplasm of the red blood cell. Therefore, a DNA-binding compound such as adozelesin needs to pen- etrate the membranes of the erythrocyte, the parasitophorous vacuole, the parasite plasma membrane and the parasite nuclear membrane before gaining access to its target. Another possi- ble explanation is that there is a threshold of DNA damage that must be irreversibly accumulated before the effects of adozelesin become cytotoxic. DNA repair pathways have not been fully elucidated in P. falciparum, but mechanisms do exist to repair certain types of DNA damage via base excision repair [27]. Perhaps a low level of DNA damage caused by a short- term treatment of adozelesin can be repaired by the parasite, but with longer exposure times, the repair machinery is over- whelmed and the parasite succumbs to the lethal effects of this DNA damage.

A surprising effect of adozelesin on malaria cultures is the failure of the parasites to arrest at the trophozoite stage, as observed following treatment with DNA replication inhibitors such as aphidicolin [25]. In mammalian cell lines and yeast, adozelesin is thought to inhibit DNA replication by physically blocking the replication machinery with drug-DNA adducts [7,22,24]. This results in the accumulation of stalled replica- tion forks and the arrest of cells during the S-phase of the cell cycle [7,23]. The presence of these stalled replication forks induces the activation of checkpoint pathways, as observed by
useful tool to study DNA damage pathways in malarial parasites. Nevertheless, this compound provides a new chemotherapy for cancer over a decade ago. Unfortunately, myelotoxicity observed in human phase II clinical trials precluded further development in the clinic [33]. While it is possible that the doses and regimens required for malaria treatment differ significantly from those used in cancer chemotherapy, the same concern applies to the use of adozelesin as an anti-malarial in humans. Nevertheless, this compound provides a useful tool to study DNA damage pathways in P. falciparum. Furthermore, this work provides proof-of-principle support for the effort to develop analogues of adozelesin that maintain the A/T-specificity and anti-malarial activity without the associated toxicity in mammals. In fact, a number of such compounds have recently been synthesized and reported to have anti-tumor activity [34]. Given our results with adozelesin in vitro and in vivo, these analogues could be equally effective against P. falciparum and represent a potential class of new highly potent and specific anti-malarials. We are currently evaluating these compounds.

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