Anti-parasitic drug discovery takes a giant leap forward

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Abstract

Although rare, parasitic infections can be severe and cause death. Presently, there is a paucity of compounds to treat these infections. Zhou et al. (1) have identified two steroidal suicide substrate inhibitors (cholesta-5,7,22,24-tetraenol (CHT)1 and ergosta-5,7,22,24(28)-tetraenol (ERGT)) directly inhibiting the sterol methyltransferase activities of Acanthamoeba castellanii (AcSMTs), the organism causing blinding keratitis (BK) and granulomatous amebic encephalitis (GAE). They demonstrated that these steroids 1) covalently bound and inhibited sterol C28-methyltransferase (Ac28-SMT), 2) were highly growth inhibitory to trophozoite growth (IC50~nM), and 3) were nontoxic to mammalian cells. The future validation of these structures as bone fide anti-parasitic therapeutics will spark great interest in the future targeting of sterol biosynthesis for treating parasitic infections.

Acanthameoba spp. have a rudimentary life cycle consisting of trophozoite and cyst stages (Fig. 1). The trophozoite phase predominates when nutrients are plentiful, while nutrient depletion or drug treatment drives the initiation of the cyst phase (2). Trophozoites infect human hosts and are disease spreading, while the cyst phase is dormant and protects the organism against host responses. Anti-Acanthameoba treatments are severely limited and include milfetosine, biguanide, and voriconazole (3).

The Acanthameoba spp. sterol biosynthetic pathway uses cycloartenol as the precursor for C28-ergosterol and C29-7-dehydroergosterosterol end product synthesis, rather then lanosterol that is used by Trypanosoma brucei (Fig. 1). The sterol 24- and C28-methyltransferases (Ac24-SMT & Ac28-SMT) are intricately involved in terminal synthesis. Ac24-SMT uses cycloartenol to synthesize 24-methylene cycloartenol, the precursor for the synthesis of the Ac28-SMT substrate, 24(28)-methylene Lophenol. This intermediate sits at a branch point, as it can be used as a precursor for ergosterol synthesis or shunted towards C29-7-dehydroergosterosterol formation. Both Acanthameoba SMTs are more orthologous to plant SMT enzymes, rather then those of trypanosomes. This sets up the unique opportunity for species-specific anti-ameobic treatment.

Using a number of elegant kinetic and in vivo cell culture studies, in association with inhibitor-product analysis, Zhou et al., (1) extensively interrogated the steroids cholesta-5,7,22,24-tetraenol (CHT) and ergosta-
5,7,22,24(28)-tetraenol (ERGT) as to their potential ability to target and inhibit Ac24-SMT & Ac28-SMT (Fig. 1). In total, this work has definitively established CHT and ERGT as steroidal suicide substrates of Ac28-SMT that can inhibit trophozoite cell growth with high potency.

The authors started off their journey by asking the important question of whether CHT and ERGT were worth pursuing as steroidal inhibitors. They tested if these steroids inhibited trophozoite growth in cell culture. Compounds were tested against Acanthameoba castellanii trophozoites. Importantly, they found that CHT and ERGT were highly potent inhibitors with IC$_{50}$ and MAC values of 51nM and 5µM, respectively. Wash out experiments, whereby each steroid was removed after a period of time and growth was then monitored, showed that trophozoites were still unable to grow even in the absence of either steroid, giving the authors the first hint that CHT and/or ERGT may act as suicide substrate inhibitors. Finally, they found that neither steroid was cytotoxic to HEK293 mammalian cells.

Based on these very positive results, they next performed elegant in vitro enzymatic assays, linked to extensive GC/MS product identification, to both characterize the CHT- and ERGT-derived products formed, and gain insight into their mechanisms of action. Ac24-SMT was able to convert CHT to the single product, ERGT, whereas ERGT itself did not productively bind to the enzyme. On the other hand, Ac28-SMT converted CHT to multiple sterol species, including C28 and C29 sterols, C28 and C29 monols, and C28- C29-steroidal diols. Overall, the types of products formed supported the hypothesis that they act as suicide substrates, forming an irreversible covalent complex with Ac28-SMT. The authors postulated that the covalent interaction was stabilized by the conjugated double bond in each analog.

Next, the authors used site-directed mutagenesis and converted the conserved Tyr60 and Tyr64 residues found within region 1 of Ac24-SMT and Ac28-SMT, respectively, to explore mechanistically their importance in appropriate substrate binding and product formation. Each Tyr was converted to a Phe or Leu, and products were identified using CTO (cholesta-5,7,24-trienol), CHT and ERGT as substrates. CTO is used as an in vitro and in vivo and served as a control substrate for product formation.

Interestingly, mutation of Ac24-SMT Tyr60 to either Phe or Leu did not alter the product species formed using CTO or CHT as substrates, when compared those produced by wild type Ac24-SMT. On the other hand, mutating Tyr64 to Phe within Ac28-SMT caused dramatic shifts in the ratios of products formed using CTO as a substrate. The data supported the idea that Tyr64 was essential for the sequential first and second C1 transfer reaction. Products formed when CHT or ERGT were used as substrates showed a severe reduction in the products that would be seen if Ac28-SMT was active. The substitution of Leu total abolished activity, as ERGT was not converted to any product(s).

Finally, they obtained more direct evidence that CHT and ERGT were irreversible inhibitors, evidenced by the fact that when CHT or ERGT were used as substrates for Ac28-SMT, the products formed were species that would be seen only if the methylation reactions were inhibited. $K_m$ and $k_{cat}$ values obtained for CHT were similar to those of the natural substrate, methylenephenol. They also showed that CHT had IC$_{50}$ and $k_i$ values for Ac28-SMT that were similar to those seen for related SMTs. Finally, they went on to show that high concentrations of methylenephenol protected Ac28-SMT from inactivation, further validating the hypothesis of an irreversible mechanism of action occurring at the active site.

The Nes laboratory has had a long-standing expertise in identifying and biochemically characterizing sterol biosynthesis inhibitors targeted against many pathogenic microorganisms (4-7). In fact, they recently characterized steroidal transition state and suicide substrate inhibitors targeting SMTs (24(R,S),25-epiminolanosterol (EL) and 26,27-dedehydrolanosterol (DHL), respectively) (8). These past studies have laid the foundation for the present work described in Zhou et al., (1), whereby two novel suicide substrate inhibitors have been studied as potential efficacious anti-parasitic therapeutics.

Based on work presented by Zhou et al., (1) these steroids can now be added to the ever-
growing inhibitor catalog of the Nes laboratory. The important work in Zhou et al., (1) will undoubtedly increase the optimism of these steroidal inhibitors becoming bona fide anti-parasitic therapeutics in the near future. Results showing their efficacy in murine infection models would seal the deal.

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Abbreviations: IC_{50}, inhibitor concentration giving 50% cell death; MAC, minimal amoebicidal concentration; BK, blinding keratitis; GAE granulomatous amebic encephalitis; SMT sterol methyltransferase; Ac Acanthameoba; CTO, cholesta-5,7,24-trienol; CHT, cholesta-5,7,22,24-tetraenol; ERGT, ergosta-5,7,22,24(28)-tetraenol.

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Fig. 1. *Acanthameoba castellanii* life cycle and sterol biosynthetic pathway. *Acanthameoba castellanii* can exist as a trophozoite when nutrients are plentiful, or upon nutrient depletion a dormant cyst. Trophozoites are the active disease spreading stage. Trophozoite sterol synthesis proceeds by way of the conversion of the precursor cycloartenol to the end products, ergosterol or 7-dehydroporiferasterol. Cycloartenol is converted to 24-methylene cycloartenol by the *Ac*24-SMT. *Ac*28-SMT converts methylene Lopenol to 24(28)E-ethylidene Lopenol. Methylene Lopenol is a branch point sterol used for the production of terminal sterols. Both SMT reactions are inhibited by CHT (1) and ERGT (2).

*Figure adapted from* (9).
**Host Invasion**

- **Trophozoite**

**Nutrient Deprivation**

- **Drug Treatment**

**Cyst**

**Dormancy**

**Ent Deprivation JG Treatment**

- **Abundant Nutrients**

**Cycloartenol**

- **Ac24-SMT**

**24-Methylene Cycloartenol**

- **Cholesta-5,7,22,24-Tetraenol (CHT)**

**Ergosta-5,7,22,24(28)-Tetraenol (ERGT)**

**24(28)-Methylene Lophenol**

- **Ac28-SMT**

**24(28)-E-Ethylidene Lophenol**

**Ergosterol**

- **7-Dehydroporiferasterol**