commentary

Anti-parasitic drug discovery takes a giant leap forward[1]

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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) and ergosta-5,7,22,24(28)-tetraenol (ERGT)] directly inhibiting the sterol methyltransferase activities of *Acanthamoeba castellanii* (*Ac*SMTs), the organism causing blinding keratitis (BK) and granulomatous amebic encephalitis (GAE). They demonstrated that these steroids *1*) covalently bound and inhibited sterol C28-methyltransferase (*Ac*28-SMT), *2*) were highly growth inhibitory to trophozoite growth (IC_{50}~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.

Acanthamoeba spp. are ubiquitous free-living protozoa causing diseases of the eye, skin, and central nervous system (2). *Acanthamoeba castellanii* causes BK and GAE. Individuals with GAE are immune-compromised and although infections are rare, a high mortality rate is seen. The fact that *Acanthamoeba spp.* infections are on the rise clearly reinforces the need for drug discovery aimed at improving the production of novel and highly potent anti-parasitic therapeutics.

Acanthamoeba 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-*Acanthamoeba* treatments are severely limited and include milfetosine, biguanide, and voriconazole (3).

The *Acanthamoeba spp.* sterol biosynthetic pathway uses cycloartenol as the precursor for C_{28}-ergosterol and C_{29}-7-dehydroporiferasterol end-product synthesis, rather then lanosterol that is used by *Trypanosoma brucei* (Fig. 1). The sterol 24- and C28-methyltransferases (*Ac*24-SMT and *Ac*28-SMT) are intricately involved in terminal synthesis. *Ac*24-SMT uses cycloartenol to synthesize 24-methylene cycloartenol, the precursor for the synthesis of the *Ac*28-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 toward C29-7-dehydroporiferasterol formation. Both *Acanthamoeba* SMTs are more orthologous to plant SMT enzymes rather than 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 *Ac*24-SMT and *Ac*28-SMT (Fig. 1). In total, this work has definitively established CHT and ERGT as steroidal suicide substrates of *Ac*28-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 whether these steroids inhibited trophozoite growth in cell culture. Compounds were tested against *Acanthamoeba castellanii* trophozoites. Importantly, they found that CHT and ERGT were highly potent inhibitors with IC_{50} and minimal amoebicidal concentration values of 51 nM and 5 μM, respectively. Washout 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. *Ac*24-SMT was able to convert CHT to the single product ERGT, whereas ERGT

Abbreviations: BK, blinding keratitis; CHT, cholesta-5,7,22,24-tetraenol, CTO, cholesta-5,7,24-trienol, ERGT, ergosta-5,7,22,24(28)-tetraenol, GAE, granulomatous amebic encephalitis; SMT sterol methyltransferase.

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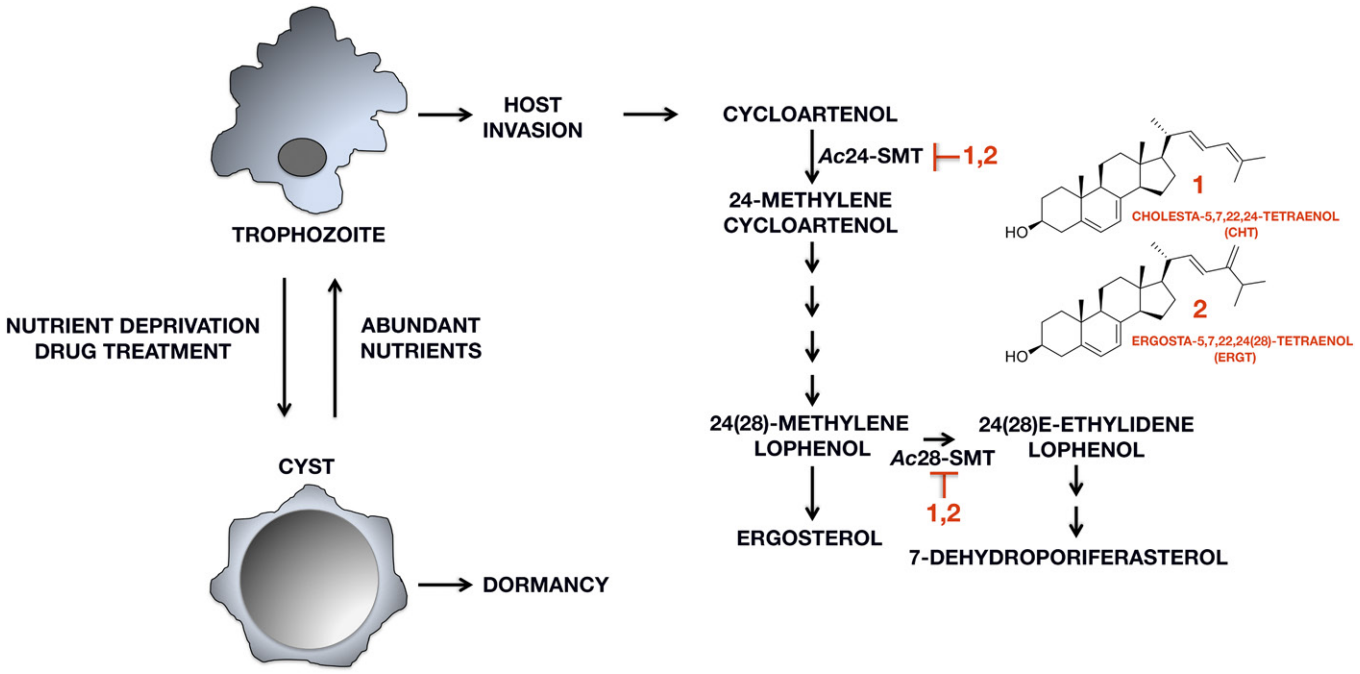

Fig. 1. *Acanthamoeba castellanii* life cycle and sterol biosynthetic pathway. *Acanthamoeba castellanii* can exist as a trophozoite when nutrients are plentiful or upon nutrient depletion, a dormant cyst. Trophozoites are the active disease-spreading stage. Trophozoite sterol synthesis proceeds by way 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 lophenol to 24(28)E-ethylidene lophenol. Methylene lophenol is a branch-point sterol used for the production of terminal sterols. Both SMT reactions are inhibited by CHT (3) and ERGT (1). Figure adapted from (9).

itself did not productively bind to the enzyme. On the other hand, *Ac*28-SMT converted CHT to multiple sterol species, including C_{28} and C_{29} sterols, C_{28} and C_{29} monols, and C_{28}- C_{29}-steroidal diols. Overall, the types of products formed supported the hypothesis that they act as suicide substrates, forming an irreversible covalent complex with *Ac*28-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 *Ac*24-SMT and *Ac*28-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 substrate and served as a control substrate for product formation.

Interestingly, mutation of *Ac*24-SMT Tyr60 to either Phe or Leu did not alter the product species formed using CTO or CHT as substrates when compared to those produced by wild-type *Ac*24-SMT. On the other hand, mutating Tyr64 to Phe within *Ac*28-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 C_1 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 totally abolished activity, as ERGT was not converted to any product(s).

Finally, they obtained more direct evidence that CHT and ERGT were irreversible inhibitors, shown by the fact that when CHT or ERGT were used as substrates for *Ac*28-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 *Ac*28-SMT that were similar to those seen for related SMTs. Finally, they went on to show that high concentrations of methylenephenol protected *Ac*28-SMT from inactivation, further validating the hypothesis of an irreversible mechanism of action occurring at the active site.

The Nes laboratory has had a longstanding 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 and 26,27-dehydrolanosterol, respectively) (8). These past studies have laid the foundation for the present work described in Zhou et al. (3), whereby two novel suicide substrate inhibitors have been studied as potential efficacious anti-parasitic therapeutics.

Based on work presented by Zhou et al. (3), these steroids can now be added to the ever-growing inhibitor catalog of the Nes laboratory. The important work in Zhou et al. (3) will undoubtedly increase the optimism

that these steroidal inhibitors will become bona fide antiparasitic therapeutics in the near future. Results showing their efficacy in murine infection models would seal the deal.

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