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Elucidating a chemical defense mechanism of Antarctic sponges: A computational study

Sai Lakshmana Vankayala[a,1], Fiona L. Kearns[a,1], Bill J. Baker[a], Joseph D. Larkin[b], H. Lee Woodcock[a,*]

[a] Department of Chemistry, University of South Florida, 4202 E. Fowler Ave., CHE205, Tampa, FL 33620, USA
[b] Department of Chemistry, Eckerd College, 4200 54th Avenue South, St. Petersburg, FL 33711, USA

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ABSTRACT

In 2000, a novel secondary metabolite (erebusinone, Ereb) was isolated from the Antarctic sea sponge, *Isodictya erinacea*. The bioactivity of Ereb was investigated, and it was found to inhibit molting when fed to the arthropod species *Orchomene plebs*. Xanthurenic acid (XA) is a known endogenous molt regulator present in arthropods. Experimental studies have confirmed that XA inhibits molting by binding to either (or both) of two P450 enzymes (CYP315a1 or CYP314a1) that are responsible for the final two hydroxylations in the production of the molt-inducing hormone, 20-hydroxyecdysone (20*E*). The lack of crystal structures and biochemical assays for CYP315a1 or CYP314a1, has prevented further experimental exploration of XA and Ereb's molt inhibition mechanisms. Herein, a wide array of computational techniques – homology modeling, molecular dynamics simulations, binding site bioinformatics, flexible receptor–flexible ligand docking, and molecular mechanics-generalized Born surface area calculations – have been employed to elucidate the structure–function relationships between the aforementioned P450s and the two described small molecule inhibitors (Ereb and XA). Results indicate that Ereb likely targets CYP315a1 by interacting with a network of aromatic residues in the binding site, while XA may inhibit both CYP315a1 and CYP314a1 because of its aromatic, as well as charged nature.

1. Introduction

Natural products research aims to isolate and characterize primary or secondary metabolites released by biological systems. Novel compounds with relevant bioactive properties are then used in pharmaceuticals, nutraceuticals, cosmetics, chemical engineering and more [1,2]. One core component of successful natural products research is to identify sources of chemical diversity that are rooted in species diversity and thus also driven by the evolutionary factors of competition, predation, and defense [1,2]. Sessile species hold unique promise for producing novel metabolites as they often rely on chemical defense mechanisms due to their lack of mobility [2]. In 2000, Baker and coworkers reported one such unique aromatic metabolite from the Antarctic sponge *Isodictya erinacea*, [1], which was named erebusinone (Ereb, Fig. 1), after the Erebus Bay where the *I. erinacea* specimen was collected.

Interest in Ereb's ecological role piqued when feeding studies performed with *Orchomene plebs* revealed Ereb inhibits molting in this small arthropod, resulting in high mortality. These observations supported the hypothesis that Ereb chemically disrupts molting in the crustacean predators of *I. erinacea* [1]. Other research has determined the effect of xanthurenic acid (XA), a known endogenous molt inhibitor, on crustacean molting [3–5] and, due to structural and functional similarities to Ereb, it was proposed the two may inhibit this pathway by the same mechanism [1,4–6]. XA is known to interact with, and inhibit, one or more P450s responsible for secretion of 20-hydroxyedysone (20*E*); however, the exact details of how XA binds to either CYP315a1 or CYP314a1, and which binding site is favored by XA, remain unclear [3–5].

The circulatory molt inducing hormone (20*E*) is produced ultimately from extracellular cholesterol through a series of successive biotransformations in various physiological locations [7,8]. The final stages of this pathway are a series of hydroxylations performed by a small set of closely related cytochrome P450 enzymes [7–9].[2] Ecdysis, or ecdysteroidogenesis, is the biochemical pathway by which ecdysteroids (molting hormones) are produced and

* Corresponding author.
E-mail address: hlw@usf.edu (H. Lee Woodcock).
[1] These authors contributed equally.

[2] Throughout this text we may refer to such closely related P450s as the "molting protein(s)" to emphasize that these are responsible for the production of the molt-inducing hormone, 20*E*.

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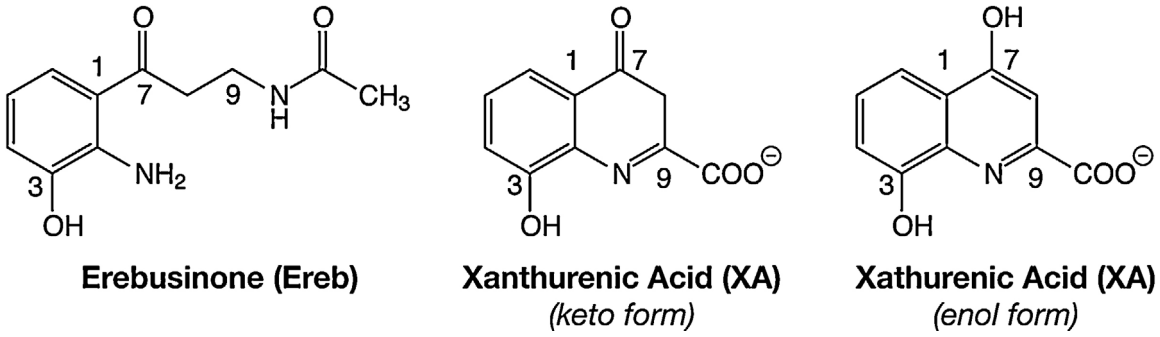

Fig. 1. Structures of the small molecule inhibitors (SMIs) investigated in this work: erebusinone (Ereb) and xanthurenic acid (XA). XA is shown here in both its keto and enol forms.

regulated for molting and growth in arthropods. This pathway has been studied thoroughly in insects, but remains somewhat clouded in the context of their crustacean cousins [10]. There are some key results, however, that may allow us to apply knowledge about insect ecdysis to crustaceans. In insects, the intermediate ecdysone (*E*) is released from the prothoracic gland (PG) to surrounding tissues where it undergoes the final hydroxylation step to produce 20*E* [9,11]. In crustaceans, *E* is secreted from the Y-organ complex (YOC) to surrounding tissues followed by hydroxylation to produce 20*E*, thusly the insect PG and crustacean YOC are often considered physiologically analogous, indicating a conservation of the pathway amongst arthropods [9,6,11–17]. It has been amply supported that the original series of hydroxylation reactions that occur in crustaceans to produce 20*E* is also highly conserved in insects [16,17].

As mentioned, enzymes responsible for the final hydroxylations to produce 20*E*, are members of a small group of cytochrome P450 enzymes (also known as CYPs or P450s). Studies performed on insect (*Drosophila* and *Bombyx*) species have provided most of our understanding about ecdysteroidogenesis and its molecular pathway [9,16,18–20]. Recent studies have sequenced various crustacean genomes and have identified orthologs to insect ecdysis P450s present in crustacean DNA, therefore the crustacean molting pathway can be appropriately modeled with insect sequences. The overall 20*E* biosynthesis begins with cholesterol and, after several steps, yields 2-deoxyecdysone (2-dE), the *E* precursor [10,16,20,21]. 2-dE is hydroxylated by the C2-hydroxylase, CYP315a1, releasing *E* which binds to CYP314a1 for hydroxylation at C_{20} finally producing 20*E* [22,23]. Past studies report that the target for regulation by XA is either CYP315a1 or CYP314a1 [2,5]. Fig. 2 highlights these final two hydroxylation steps, which are herein investigated.

The purpose of this current work is to predict how Ereb and XA inhibit 20*E* biosynthesis and thus crustacean molting [2–4]. From here on we refer collectively to Ereb and XA as "small molecule inhibitors," or SMIs, Fig. 1 compares these SMIs. A variety of computational methods including homology modeling, molecular dynamics (MD) simulations, binding mode analysis, flexible docking studies, and molecular mechanics – generalized Born surface area (MM-GBSA) calculations, have been used to investigate SMI binding in CYP315a1 and CYP314a1. Additionally, we compared the observed interactions between the SMIs and P450 binding sites to the interactions between the native substrates (2-dE, *E*, and 20*E*) and P450 binding sites to predict how SMI binding may interrupt enzymatic activity.

2. Computational details

2.1. P450 homology modeling

Query amino acid sequences of *Drosophila melanogaster* P450s, CYP315a1 and CYP314a1, were obtained from the NIH GenBank (accession numbers AAS65010.1 and AAL86019.1 respectively) [22,24]. The BLAST (basic local alignment search tool) algorithm [25] was used to perform a local structural sequence alignment search through the non-redundant PDB [26] for a template homolog, and CYP24a1 was identified (PDB code: 3K9V chain A, a rat P450 that hydroxylates vitamin D), with the highest sequence similarity for both CYP315a1 (BLAST-bit score: 173.3) and CYP314a1 (BLAST-bit score: 166.0). Homology models of the *Drosophila* molting proteins CYP315a1 and CYP314a1 were constructed using the Prime 3.1 comparative modeling module [27]. The iron protoporphyrin IX (heme) and 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CPS, the CYP24a1 substrate, Fig. SI1) from the 3K9V-A crystal structure were retained in the query binding site to aid in homology model construction. For serial loop sampling, Prime uses a hierarchical refinement protocol with a loop buildup algorithm (see Supporting Information for more details) [28]. Further, side-chain optimizations are performed on filtered loop candidates, followed by full minimizations of the loops until convergence of the gradient is reached (< 0.001 kcal mol^{-1} $Å^{-1}$). Prime uses the OPLS-2005 all atom force field [29] in conjunction with generalized Born implicit solvation (GBSA) to score the energies of loop candidates. After loop refinement, the homology models were further improved using Truncated Newton Conjugate Gradient (TNCG) minimization [30] until the RMSD of heavy atoms reached a maximum cut-off of 0.3 Åfrom their respective initial coordinate geometries. Ramachandran plots were generated using PROCHECK [31]. Finally, an online homology modeling Web-server, I-TASSER, was used to generate models for structural comparison with our models [32–34]. Homology models of CYP315a1 and CYP314a1 were then simulated as described below.

2.2. Molecular dynamics simulations

Molecular dynamics (MD) simulations were performed using the CHARMM biomolecular simulation program [35–37]. CHARMMing [38] and pdbtools (https://github.com/harmslab/pdbtools) were used to parse the homology models for correct atom types and residue numbers and to prepare the structures for simulation. The proteins were treated with the CHARMM (C22) force field, while substrate topology and parameters were evaluated using the CHARMM generalized force field (CGenFF) via the Paramchem Web-interface [39–41]. The protein structures were solvated with the addition of 46,656 modified TIP3P waters [42] in a cubic box of size of 90 Å× 90 Å× 90 Å, and Periodic Boundary Conditions (PBCs) [43] were applied with particle-mesh Ewald (PME) [44] to handle long-range electrostatic interactions (κ = 0.34, order of B-spline interpolation set to 6). Lennard–Jones interactions were treated with a heuristically updated atom-based cutoff scheme, where the non-bonding pair list was generated to include all atoms within 12 Å, and the switching function was evaluated for atoms between