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Publication Date

2013

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UNIVERSITY OF CALIFORNIA, SAN DIEGO

An Evaluation of Compounds Derived from Natural Products as Potential Activators
of Cyclic Adenosine Monophosphate Production

A Thesis submitted in partial satisfaction of the requirements
for the degree Master of Science

in

Biology

by

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Professor Martin Tresguerres, Chair
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Professor Lena Gerwick

2013

The Thesis of Jason Han-Chieh Ho is approved and it is acceptable in
quality and form for publication on microfilm and electronically:

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University of California, San Diego

2013

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LIST OF ABBREVIATIONS

ATP: Adenosine triphosphate.

BSA: Bovine serum albumin.

cAMP: Cyclic adenosine monophosphate.

COS7: African green monkey SV40-transformed kidney fibroblast cell line.

CP: Creatine phosphate.

CPK: Creatine phosphokinase.

(phospho)-CREB: (Phosphorylated)-cAMP response element binding protein.

CRP: cAMP receptor protein.

DMEM: Dulbecco's modified Eagle medium.

DMSO: Dimethyl sulfoxide.

DTT: Dithiothreitol.

EC₅₀: Half maximal effective concentration.

ELISA: Enzyme-linked immunosorbent assay.

Epac: Exchange protein directly activated by cAMP.

GLP: Glucagon-like peptide.

GPCR: G protein-coupled receptor.

HEK 293: Human embryonic kidney 293 cells.

HRP: Horseradish peroxidase.

IBMX: 3-isobutyl-1-methylxanthine.

IC₅₀: Half maximal inhibitory concentration.

KDa: Kilo Dalton.

KH7: Specific inhibitor of soluble adenylyl cyclase.

K_m : EC_{50} ; half maximal effective concentration.

LPS-free FBS: Lipopolysaccharide-free fetal bovine serum.

MAPK: Mitogen-activated protein kinase.

MEF: Mouse embryonic fibroblast cells.

NO: Nitric oxide.

PBS: Phosphate buffer solution.

PDE: Phosphodiesterase.

Pen/strep: Penicillin-Streptomycin.

PKA: Protein kinase A.

PP_i : Pyrophosphate.

P-site: P-site inhibitor of transmembrane adenylyl cyclase.

PVDF: Polyvinylidene fluoride.

$sAC_{(t/fl)}$: Soluble adenylyl cyclase (truncated or full-length protein).

SDS-PAGE: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

TBST: Tris-buffered saline with Tween 20.

tmAC: Transmembrane adenylyl cyclase.

V_{max} : Maximum rate of enzyme reaction.

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ACKNOWLEDGEMENTS

I would like to acknowledge my committee members for their unwavering support when I was preparing my Thesis and giving my defense. In particular, my mentor and graduate adviser Martin Tresguerres, encouraged me to set my own goals and matched my determination to see them fulfilled. Lena Gerwick had a lot of enthusiasm for my experiments, and was happy to let me to use the equipment and resources of her laboratory. I would also like to thank Milton Saier for his curiosity in my research, and Eric Allen for his experience with the Master's program.

I am grateful to the members of the Tresguerres laboratory for their friendship, support, and encouragement. Katie Barott and Megan Barron's work with corals provided me with essential background information, while Jenny Tu supplied me with live coral fragments for my experiments. I also want to thank Jinae Bartlett, Lauren Linsmayer and Sidney Perez for their encouragement.

The members of William and Lena Gerwick's laboratory were integral for the success of my project. Tara Byrum and Evgenia Glukhov supplied me with the pure compounds for my research. Paul Boudreau synthesized the laurencione monoacetate compound, and Sam Mascuch taught me cell culture techniques. Finally Bailey Miller, Matthew Bertin, and Emily Mevers were always willing to lend a helping hand.

I would like to thank Jochen Buck and Lonny Levin from the Wiell Cornell Medical College for their support. Lavoiser Ramos-Espiritu sent me the cell lines for my experiments, and Jacob Bitterman provided me with the purified rat sAC₁ protein.

Finally I would like to thank all of my friends and family who believed in me when I didn't even believe in myself. I managed to attain my goals with their support.

ABSTRACT OF THE THESIS

An Evaluation of Compounds Derived from Natural Products as Potential Activators
of Cyclic Adenosine Monophosphate Production

by

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Master of Science in Biology

University of California, San Diego, 2013

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The widely conserved cyclic adenosine monophosphate (cAMP) signaling pathway governs multiple physiological responses. Mammals express two types of adenylyl cyclases: transmembrane adenylyl cyclase (tmAC), and soluble adenylyl

cyclase (sAC). The distinct regulatory properties, tissue specificity and cellular distributions of the adenylyl cyclases make them attractive targets for research and drug development. While P-site inhibitors preferentially inhibit tmAC isoforms, drugs derived from catechol estrogens (4CE) and KH7 selectively inhibit sAC. All of the existing adenylyl cyclase stimulators are analogs of the tmAC activator forskolin. There are currently no pharmacological stimulators of sAC in literature. In this study, I took advantage of the high rate of cAMP production in coral tissue homogenates to screen the effects of 151 pure compounds derived from natural products. 50 μ M Laurencione monoacetate stimulated cAMP production in coral tissues by 60%, with an EC_{50} of $\sim$ 25 nM—1 μ M. The same treatment also stimulated purified rat sAC_t activity by 80%, with an EC_{50} lower than 5 nM. This effect on rat sAC_t was not evident with 25 mM HCO₃⁻. Though cAMP accumulation assays in live cells suggested cross-talk effects with laurencione monoacetate, a Western blot detecting proteins phosphorylated by PKA suggested that the compound affected intracellular PKA activity. This study demonstrates that corals are useful subjects for screening compounds that affect cAMP production, and that natural products are good candidates for drug discovery. Identification of a sAC-specific stimulator that can be used as a pharmacological tool is challenging due to the complexity of the cAMP signaling pathway.

INTRODUCTION

Cyclic adenosine monophosphate (cAMP) is a widely conserved messenger molecule used to mediate cellular responses to a variety of external signals. It is involved in a number of signaling cascades, and regulates specific physiological responses that differ by species, tissue types, and even by sub-cellular compartments. Due to this wide distribution, cAMP signaling cascades are kept under tight spatial and temporal regulation (DiPilato et al., 2004). Furthermore, their roles in governing important physiological responses make them attractive targets for research and drug development. In mammals, adenosine triphosphate (ATP) is converted into cAMP by two types of adenylyl cyclases: transmembrane adenylyl cyclase (tmAC), and soluble adenylyl cyclase (sAC).

Transmembrane adenylyl cyclases are membrane-associated enzymes present in all animals that are regulated by hormones and neurotransmitters via heterotrimeric G proteins (reviewed in Kelly et al., 2008; Sadana and Dessauer, 2009). Nine different isoforms of mammalian tmAC have been characterized (tmAC I-IX), each of which can be regulated by different G protein-couple receptors (GPCR) and expressed in specific tissues (reviewed in Pierre et al., 2009).

By contrast, soluble adenylyl cyclase is evolutionarily, molecularly and biochemically distinct from the tmAC isoforms. Molecular analysis revealed that mammalian sAC is more closely related to cyanobacterial adenylyl cyclases than to mammalian tmACs, and biochemical assays demonstrated that it was uniquely stimulated by HCO_3^- (Buck et al., 1999; Chen et al., 2000). Mammalian sAC was first discovered in rat testes, where it was necessary for fertilization by regulating sperm

capacitation and flagellar motility (Hess et al., 2005). Identification of sAC in somatic tissues suggests potential roles in multiple physiological processes (reviewed in Tresguerres et al., 2011). For example, cAMP production by sAC was necessary for insulin secretion in pancreatic cells (Zippin et al., 2013). The soluble nature of sAC allows it to function in cellular compartments distinct from the plasma membrane, such as the mitochondria (Zippin et al., 2003). Resident pools of sAC were also discovered in the nucleus, where it is suggested to regulate gene transcription via PKA and CREB (Zippin et al., 2004).

A major downstream effect of cAMP is the phosphorylation of proteins by protein kinase A (PKA). When cAMP binds to the regulatory subunit of PKA, the catalytic domain becomes free to phosphorylate target proteins at a conserved serine/threonine consensus sequence (Kim et al., 2007; Das et al., 2007). Phosphorylation can either stimulate or inhibit a target protein's activity. Some of the targets for PKA are other kinases, such as the mitogen-activated protein kinase (MAPK), which in turn phosphorylates more proteins in a cascade for rapid signal amplification (Cao et al., 2001). PKA is expressed in most cellular compartments, including the nucleus where it can directly activate the cAMP response element-binding transcription factor (CREB) to regulate gene expression (reviewed in Mayr and Montminy, 2001). PKA can also regulate its own activity by activating phosphodiesterases (PDE) which degrade cAMP, creating a negative feedback loop (Sette and Conti, 1996). Another way that PKA regulates cAMP signals is by phosphorylation and desensitization of the G protein-coupled receptors (reviewed in

Kelly et al., 2008). Phosphorylated receptors are uncoupled from their G proteins by Arrestin, preventing further stimulation of tmACs (Penn et al., 2000).

Exchange proteins activated by cAMP (Epac) make up another class of cAMP-dependent effectors that regulate cell physiology. The targets of Epac include the Ras family of GTPases (Rap1 and Rap2), which affect processes that determine cell fate, including cell differentiation and the formation of cell junctions (reviewed in Cheng et al., 2008; Gloerich and Bos, 2010). Hormonal signals relayed by tmACs are likely responsible for these irreversible processes. Insulin secretion in pancreatic cells is a sAC-dependent process that is regulated by Epac (reviewed in Cheng et al., 2008; Gloerich and Bos, 2010; Zippin et al., 2013).

Finally, the activities of certain cyclic nucleotide-gated ion channels involved in cardiac and olfactory functions are sensitive to cAMP. In the heart, pacemaker ion channels bound by cAMP require less voltage for activation (reviewed in Gancedo, 2013). Meanwhile, cAMP can directly modify channel activities in olfactory neurons, allowing passage of Na^+ , K^+ and Ca^{2+} ions (reviewed in Gancedo, 2013). Divalent cations such as Mg^{2+} and Ca^{2+} are often cofactors that affect adenylyl cyclase activity. Ca^{2+} ions in particular can stimulate mammalian sAC, while the numerous isoforms of mammalian tmAC are more varied in their response (reviewed in Willoughby and Cooper, 2007; Litvin et al., 2003; Zippin et al., 2013; Tresguerres et al., 2011).

Cyclic AMP signaling pathways need to be tightly regulated within a cell, since they are involved in regulating multiple cellular processes. While adenylyl cyclases produce cAMP, enzymes called phosphodiesterases (PDE) degrade it in order to limit the range of the cAMP signals to microdomains within cells (reviewed in

Tresguerres et al., 2011). The diverse distribution and functions of the cAMP signaling pathways make them an attractive subject for pharmacological studies. Research in drug development would ideally target cAMP signals in a tissue-specific and cellular microdomain-specific manner.

Drugs that target the G protein-coupled receptors for their ligand specificity are not useful in long-term treatments, due to GPCR desensitization by Arrestin (reviewed in Kelly et al., 2008; Penn et al., 2000). PDE inhibitors are used to prevent cAMP degradation (reviewed in Pierre et al., 2009), but cannot discern the differences between the specific adenylyl cyclase isoforms. Recent insight on the distinct regulatory properties, tissue specificity and cellular distributions of the different adenylyl cyclases has generated rising interest for the development of isoform-specific drugs. The tmAC isoforms V and VI are popular subjects for pharmacological research due to their involvement in regulating cardiac activity (reviewed in Willoughby and Cooper, 2007). Putative functions for sAC include cellular pH regulation and regulation of gene transcription (reviewed in Tresguerres et al., 2010), making it another interesting target for pharmacological study.

Among the various types of drugs that specifically inhibit adenylyl cyclases are compounds that mimic the ATP substrate. All adenylyl cyclases utilize this molecule, so analogous compounds tend to be non-specific inhibitors that competitively bind to the catalytic site (reviewed in Seifert et al., 2012). The P-site inhibitors (P-site) are another class of nucleotide analogs that bind to the catalytic sites of adenylyl cyclases. However instead of competing with substrate binding, P-site compounds inhibit AC activity by stabilizing the enzyme-product complex, preventing the release of

pyrophosphate (PP_i) (Dessauer and Gilman, 1997). While capable of inhibiting all AC isoforms, P-site binds to tmAC with much higher affinity (IC₅₀=8 μM) than to sAC (IC₅₀=500 μM) (Bitterman et al., 2013).

Derivatives of catechol estrogens (CE) can be adenylyl cyclase inhibitors that bind allosterically to a hydrophobic patch near the catalytic site (Steegborn et al., 2005 A). Inhibition by CE-type compounds involves chelating the Mg²⁺ cofactor necessary for AC function (Steegborn et al., 2005 A). Though capable of inhibiting tmAC activity (IC₅₀=100 μM), analogs of CE were found to inhibit sAC with much higher affinity (IC₅₀=10 μM) (Schlicker et al., 2008; Bitterman et al., 2013). Finally, the novel compound KH7 was recently characterized as a sAC-specific inhibitor (IC₅₀=10 μM) (Hess et al., 2005). While its mechanism of inhibition is still unresolved, experiments confirmed that tmAC activity was unaffected by up to 500 μM KH7 (Hess et al., 2005; Bitterman et al., 2013).

All of the activators currently characterized for adenylyl cyclases are analogs of the natural product forskolin. Forskolin is a general tmAC activator which stimulates 8 of the 9 mammalian tmAC isoforms by binding a conserved hydrophobic pocket to induce catalytic activity (Tang and Hurley, 1998). Variations in the hydrophobic pocket between the tmAC isoforms have led to the development of forskolin analogs that exhibit greater isoform specificity (Pinto et al., 2008). However, research on cAMP production in non-mammalian systems revealed that the tmACs from invertebrates such as corals were insensitive to forskolin (Barott et al., 2013). Drugs that can stimulate non-mammalian tmACs may require identifying structurally novel compounds.

No pharmacological compounds that specifically activate sAC have been characterized yet. When it was first isolated, sAC was determined to be insensitive to both G protein regulation and forskolin treatment (Buck et al., 1999; Chen et al., 2000). Physiological stimulators of sAC include HCO_3^- and Ca^{2+} (Chen et al., 2000; Willoughby and Cooper, 2007; Litvin et al., 2003), but their ionic nature means that they cannot readily pass through cellular membranes. The purpose of this study was to evaluate structurally unique compounds derived from natural products as pharmacological tools for studying adenylyl cyclase activity, with the intent of identifying a novel activator of sAC.

MATERIALS AND METHODS

Pure compounds.

The drugs under investigation were obtained from a library of 151 compounds provided by the Gerwick laboratory at the Scripps Institution of Oceanography. Compounds were either natural products purified from marine cyanobacteria and algae, or were synthetic analogues derived from these compounds. The three drugs chosen for more detailed studies were laurencione monoacetate, halogenated chamigrene, and 4S, 5R-antillatoxin A. Isolation of the laurencione natural product is described in Bernart et al., (1992), and synthesis of the monoacetate analogue used for this study is reported in Gerwick et al., (2012). The halogenated chamigrene compound investigated in this study was isolated by Howard and Fenical, (1975), while the structure and toxicity of 4S, 5R-antillatoxin A was first described by Orjala et al., (1995).

Specimens.

Pocillopora damicornis tissue homogenates were prepared from coral fragments obtained from the Birch Aquarium at Scripps. *Acropora yongei* fragments were obtained from Dimitri Deheyn's laboratory at the Scripps Institution of Oceanography. While the *P. damicornis* fragments were taken from different colonies, the *A. yongei* fragments were genetic clones of one another. Coral tissue was removed from the skeleton and homogenized using an airbrush (80 psi) in buffer containing 100 mM Tris (pH 7.5) and protease inhibitor cocktail (Sigma-Aldrich). In the case of the *A. yongei* tissue samples, mucus was broken down by sonication (three pulses lasting five seconds each, on ice). Homogenized coral tissue was

centrifuged at 500 x g for five minutes to pellet zooxanthellae, and the remaining supernatant was divided into aliquots and stored at -80°C . All cAMP assays were performed with either freshly homogenized coral tissue, or with first thaw homogenates, since samples that had been frozen and thawed more than once lost HCO_3^- stimulation of cAMP production.

An aliquot of rat sAC_i protein was obtained from Lonny Levin and Jochen Buck's laboratory at the Weill Cornell Medical College. Methods of protein expression and purification can be found in a paper referenced here (Bitterman et al., 2013).

Frozen seed cultures of 4-4 human embryonic kidney (HEK293) cells over expressing sAC_i, mouse embryonic fibroblast (MEF) wild type cells, and immortalized sAC knock out MEF cells were also obtained from Lonny Levin and Jochen Buck's laboratory at the Weill Cornell Medical College. Methods of stable transfection of the sAC_i gene into the HEK293 cells, and methods of sAC gene knock out in the MEF cells can be found in a paper referenced here (Bitterman et al., 2013).

Cell culture techniques.

Seed cultures of 4-4 HEK293, MEF WT and MEF KO cells were briefly thawed and suspended in 10 mL of culture media (Dulbecco's modified eagle media (DMEM) supplemented with 10% lipopolysaccharide-free fetal bovine serum (LPS-free FBS), 100 $\mu\text{g}/\text{mL}$ penicillin streptomycin (pen/strep), and 0.15% HCO_3^-) immediately upon reception. Suspended cell cultures were centrifuged at 300 x g for 10 minutes, and the media aspirated to remove excess dimethyl sulfoxide (DMSO).

Cell pellets were re-suspended in culture media and allowed to grow at 37 °C, and 5% CO₂.

Cell cultures were diluted into fresh media when they became ~80% confluent, which happened roughly every three days. Since all cell lines are naturally adherent, cultures could be washed with 1 x phosphate buffered saline (PBS) without dislodging cells. Suspension of cells required a 5 minute treatment with 2mL of 0.25% Trypsin in 1 x PBS, followed by vigorous rinsing with 10 mL of fresh media. The concentrated suspension of cells was then diluted 1/10 in fresh media, and incubated at 37 °C, and 5% CO₂. Cell cultures were split at least twice before use in cAMP production assays or for creating new frozen seed stocks.

To prepare frozen stocks of each cell line, a hemocytometer was used to calculate the concentration of cells in suspension during a split. Each frozen stock consisted of ~1,000,000 cells, which were pelleted at 600 x g for 10 minutes, and re-suspended in 500 µL of culture media. Equal volumes of freezing solution (50% DMEM culture media, 30% LPS-free FBS, and 20% DMSO) were added drop-wise, with gentle mixing to prevent the cells from undergoing osmotic shock from the DMSO. Cells were chilled for 30 minutes in ice, then transferred to pre-chilled cryovials in a pre-chilled freezer box lined with cotton. Cells were frozen at -80 °C overnight, then transferred to liquid nitrogen for long-term storage.

Assays for measuring cAMP production.

Total protein concentrations in samples of homogenized coral tissue were measured by the Bradford method in order to standardize cAMP production. Values of total protein concentration were measured with three to four replicates per coral tissue

sample. The total protein concentration in the aliquot of purified rat sAC_t was measured with two replicates, due to the limited volume of rat sAC_t available.

Coral tissue homogenates were mixed with an adenylyl cyclase buffer containing 100 mM Tris (pH 7.5), 0.5 mM isobutylmethylxanthine (IBMX), 0.5 mM dithiothreitol (DTT), 20 mM creatine phosphate (CP), 100 U mL⁻¹ creatine phosphokinase (CPK), 5 mM MgCl₂, and 5 mM ATP for 30 minutes at room temperature on an orbital shaker (300 rpm). In preliminary experiments, pure compounds from the Gerwick laboratory's library were tested as single replicates against a DMSO solvent control in order to screen for activators and inhibitors of cAMP production. Treatments were then performed in triplicate to generate dose response curves for the potential activators. Treatments included a solvent control (DMSO), 20 mM NaHCO₃, and increasing concentrations of pure compound (laurencione monoacetate, halogenated chamigrene, or 4S, 5R-antillatoxin A). Reactions were stopped with the addition of an equal volume of 0.2 N HCl, and were stored frozen at -20 °C.

cAMP accumulation assays with purified rat sAC_t were performed in a similar manner as the coral tissue homogenates. However, unlike tissue homogenates, the risk of degradation of substrate ATP by ATPases and product cAMP by phosphodiesterases (PDE) was not a factor with purified rat sAC_t protein. Therefore, the assay buffer did not contain IBMX, CP or CPK.

Production of cAMP in mammalian cells was standardized by cell density rather than by total protein concentration. 24-well plates were loaded with 250,000 cells well⁻¹, and allowed to incubate at 37 °C, 5% CO₂ overnight until the wells

became ~70% confluent. Media was refreshed an hour before use in experiments, and any drug that required pre-incubation (KH7 or P-site) was added. Treatments were performed with two to three replicates, and included solvent controls (DMSO), 0.5 mM IBMX, increasing concentrations of pure compound, 100 μ M forskolin, 30 μ M KH7, or 50 μ M P-site. After 20 minutes of treatment at 37 °C, 5% CO₂, reactions were quenched by aspiration of the reaction media, followed by the addition of 200 μ L of 0.1 N HCl. Reaction plates were stored frozen at –20 °C.

Concentrations of cAMP from all cAMP accumulation assays were measured by enzyme-linked immunosorbent assays (ELISA) using the acetylated protocol of the Direct cAMP ELISA kit (Enzo Life Sciences and Arbor Assays).

Western Blots.

After splitting mammalian cell cultures, remaining cells were collected by centrifugation at 600 x g for 10 minutes, and suspended in 1 mL of DMEM culture media. Treatments including a DMSO solvent control, 100 μ M forskolin, or 50 μ M of pure compound (laurencione monoacetate, halogenated chamigrene, or 4S, 5R-antillatoxin A) were added to cell suspensions and incubated at 37 °C, 5% CO₂ for 30 minutes. Following treatment, cells were centrifuged at 600 x g for 10 minutes, and the reaction media aspirated. Cell pellets were snap-frozen in liquid nitrogen, and stored at –80 °C.

Cell pellets were suspended in 50 μ L of lysis buffer containing 25 mM Tris (pH 7.5), 150 mM NaCl, 1% TritonX-100, protease inhibitor cocktail (Sigma-Aldrich), and phosphatase inhibitor cocktail (Roche), then lysed by running cells 12 times through a 26-27 G syringe needle. HEK cells were diluted 1/50—1/100, while

MEF cells were diluted 1/10—1/20 with lysis buffer. Total protein content of cell lysates was measured by the method of Bradford in order to load SDS-polyacrylamide gels with equal amounts of protein for each cell type and treatment. Before loading gels, cell lysates were diluted with equal volumes of sample buffer (Laemmli buffer with 5% β -mercaptoethanol), and heated at 70 °C for 15 minutes. Proteins in cell lysates were separated by molecular weight with SDS-PAGE, and transferred to polyvinylidene fluoride (PVDF) membranes for 30 minutes at 6 V with an electrophoretic transfer apparatus (Bio-Rad). Blots were incubated in blocking buffer (0.05 g/mL milk in TBST) for an hour with gentle agitation at room temperature. Blots were then incubated in phospho-(Ser/Thr) PKA substrate antibody (1:1000, Cell Signaling) with gentle agitation at 4 °C overnight. The next day, blots were washed three times for 15 minutes with TBST, and incubated in secondary antibody (goat anti-rabbit HRP conjugate, (Bio-Rad), 1:10,000) for an hour with gentle agitation at room temperature. After three more washes with TBST, Western blots were imaged for chemi-luminescence with substrate solution (equal parts peroxide and luminol solution) using the ImageLab software from Bio-Rad.

RESULTS

Homogenized tissue samples from the coral species *Pocillopora damicornis* were used to screen a library of pure compounds for either stimulatory or inhibitory effects on cAMP production. Coral tissues were used in these preliminary assays because they produce cAMP at very high rates (Barott et al., 2013), which facilitates measurement of cAMP production and suggests robust expression of adenylyl cyclase enzymes. This activity was stimulated by HCO_3^- and inhibited by KH7, providing evidence that corals express sAC activity (Barott et al., 2013). Differences in cAMP concentrations between the control and treatments would identify compounds capable of affecting cAMP production. As a preliminary experiment, drugs were tested without replicates against a basal level of cAMP produced by coral tissues treated with DMSO (figure 1). It was necessary to establish this control because all of the pure compounds were dissolved in DMSO solvent. The basal rate of cAMP production in *P. damicornis* tissues was determined as the mean of two values, with the first and second standard deviations calculated as a measure of variability. Pure compounds were tested at a reaction concentration of 10 $\mu\text{g/mL}$, so the molar concentrations for each compound varied depending on their molecular weights.

The preliminary screen identified a number of promising activators and inhibitors of cAMP production in corals. Only the most potent activators and inhibitors were selected for further study. Information about each compound of interest was briefly summarized in figures 2 and 3; and tables 1 and 2. The structures of the drugs known to affect adenylyl cyclase activity are also included in these figures for comparison.

The potential activators identified from the preliminary assay included laurencione monoacetate, halogenated chamigrene, and 4S, 5R-antillatoxin A (figure 2B). Laurencione monoacetate is a synthetic compound derived from the laurencione natural product, which was extracted from the red alga *Laurencia spectabilis* (Gerwick et al., 2012). The original natural product shared structural similarities with acyl homoserine lactones, and induced bioluminescence in the marine bacterium *Vibrio harveyi* (Lowery et al., 2005). This suggests that laurencione may be involved in regulation of quorum sensing and interkingdom signaling between algae and bacteria. This compound was also suggested to have anti-inflammatory activity because it inhibited nitric oxide (NO) production in macrophages exposed to bacterial lipopolysaccharides (Gerwick et al., 2012). Halogenated chamigrene is another potential activator produced by the red algae *Laurencia* (Suzuki et al., 1979). Though little is known the compound under investigation, chamigrene derivatives with structural similarities were shown to have antibacterial properties (Bansemir et al., 2004). Finally 4S, 5R-antillatoxin A, isolated from the marine cyanobacterium *Moorea producens*, was demonstrated to be a potent ichthyotoxin that activates voltage-gated Na⁺ channels (Li et al., 2001). Though this assay identified the 4S, 5R-stereoisomer as a potential activator of cAMP production in coral tissue homogenates, the 4R, 5R-stereoisomer is recognized as the dominant configuration, and is 25-fold more potent as an ichthyotoxin (Li et al., 2004). None of these compounds shared common structural motifs with forskolin, and may affect cAMP production by means independent of tmAC.

The potential inhibitors of cAMP production included scytonemin, S-4-benzyl-3-isobutyryloxazolidin-2-one, constanolactone E methyl acetonide, and malhamensilipin A (figure 3B). Scytonemin acts as a sunscreen pigment by absorbing UV-radiation in cyanobacteria (Jones et al., 2011). On the other hand, no other biological functions for either S-4-benzyl-3-isobutyryloxazolidin-2-one or constanolactone E methyl acetonide have been characterized yet. Malhamensilipin A is an antimicrobial compound from the chrysophyte *Poterioochromonas malhamensis* that inhibits the pp60 protein tyrosine kinase and shows promise as an anticancer drug (Chen et al., 1994). None of these compounds share structural motifs with the adenylyl cyclase inhibitors currently characterized, though both constanolactone E methyl acetonide and malhamensilipin A have lipid-like structures with long hydrocarbon chains.

Subsequent research focused on confirming the proposed stimulatory effects of laurencione monoacetate, halogenated chamigrene, and 4S, 5R-antillatoxin A on cAMP production in coral tissue homogenates. Dose response effects of laurencione monoacetate were first tested in four separate fragments of *Pocillopora damicornis* (figure 4), in order to replicate the stimulation observed in the preliminary assay. A 57% stimulation of basal cAMP production was observed with 50 μ M treatment, though this response variable ($p=0.323$, paired t-test). The dose response had a half maximal effective concentration (EC_{50}) of ~ 1.5 μ M laurencione monoacetate. Samples treated with both 20 mM HCO_3^- and laurencione monoacetate exhibited similar trends. HCO_3^- alone increased the basal rate of cAMP production by 36% ($p=0.129$, paired t-test), suggesting mild sAC activity in the corals.

The remaining assays with corals used tissue homogenates from *Acropora yongei*. Assays were either run the same day as tissue sampling, or with first-thaw samples. The effects of laurencione monoacetate on five coral fragments from *A. yongei* (figure 5) were consistent with the dose response observed in *P. damicornis*. For example, the basal rate of cAMP production in *A. yongei* was stimulated 56% with 50 μ M treatment ($p=0.215$, paired t-test). Samples treated with 20mM HCO_3^- also exhibited a 36% stimulation relative to the DMSO control ($p=0.108$, paired t-test). On the other hand, the EC_{50} of the dose response in *A. yongei* was lower at ~ 25 nM laurencione monoacetate. Additionally, overall cAMP production rates in *A. yongei* tissue homogenates were ~ 50 times lower than in *P. damicornis* (figures 8 and 9). The basal rate of cAMP production in *A. yongei* was $\sim 700 \text{ pmol} \cdot \text{mg}_{\text{prot}}^{-1} \cdot \text{min}^{-1}$, while *P. damicornis* produced cAMP at $\sim 34,000 \text{ pmol} \cdot \text{mg}_{\text{prot}}^{-1} \cdot \text{min}^{-1}$.

Dose response effects of halogenated chamigrene were also tested on *A. yongei* tissue homogenates (figure 6). However, instead of the expected stimulatory effect, treatment actually inhibited basal cAMP production in *A. yongei*. Tissue homogenates produced only 75% as much cAMP as the DMSO control when treated with 50 μ M of halogenated chamigrene, with an IC_{50} at ~ 6 nM. The significance of this inhibitory effect could not be determined with only two replicates. Inhibition was not observed in the presence of 20 mM HCO_3^- , but because HCO_3^- did not stimulate activity relative to the DMSO control, it was unclear if this compensation was due to sAC. Additional replicates with halogenated chamigrene in *A. yongei* were not attempted because the compound did not produce the expected stimulatory effect.

Cyclic AMP production in *A. yongei* was not affected by 4S, 5R-antillatoxin A alone (figure 7), though a 25% stimulation was observed with both 50 μ M treatment and 20 mM HCO_3^- ($p=0.018$, paired t-test). This response suggested that the drug could affect sAC activity, however the relative stimulation was too low to be of much significance. The EC_{50} for this HCO_3^- -dependent response was $\sim 1.5 \mu\text{M}$ 4S, 5R-antillatoxin A. Unfortunately, HCO_3^- alone failed to stimulate the basal rate of cAMP production, so the activity of sAC in these assays was ambiguous. Additional replicates for this experiment were not completed in favor of testing the compounds of interest with purified rat sAC_t protein.

Drugs were first tested with homogenized coral tissue because their ability to produce high concentrations of cAMP was hypothesized to make pharmacological stimulation/inhibition of cAMP production more apparent. However, with all of the native coral proteins taken out of their physiological context and mixed together in a buffered solution, it was impossible to tell if the drugs were targeting a specific aspect of the cAMP signaling pathway. The catalytic domains of coral sAC were expressed and purified by a colleague in order to measure the effects of the drugs on coral sAC_t. However, inconsistent stimulation with HCO_3^- raised questions about the integrity of its activity. The drugs were instead tested with samples of purified rat sAC_t, obtained from Lonny Levin and Jochen Buck's laboratory at the Weill Cornell Medical College.

Cyclic AMP production rates by rat sAC_t were stimulated 4-fold with 25 mM HCO_3^- ($p<0.0001$, paired t-test), confirming its activity as a HCO_3^- -dependent adenylyl cyclase. Dose response effects of laurencione monoacetate on this protein

revealed an 80% stimulation of basal activity with 50 μ M treatment ($p=0.0296$, paired t-test). The EC_{50} for this dose response was lower than 5 nM, demonstrating that laurencione monoacetate affects sAC activity with very high affinity. Laurencione monoacetate has great potential as a novel activator of mammalian sAC, as it appears to elicit a significant (yet moderate) stimulation of basal rat sAC_t activity with high affinity (figure 10). However, this effect was not evident in the presence of 25 mM HCO_3^- .

Dose response effects for the other two compounds represent single replicates, due to the limited amount of rat sAC_t remaining for reactions. Only the DMSO and HCO_3^- controls were run with multiple reactions per replicate in order to confirm the sensitivity of rat sAC_t to HCO_3^- . Neither halogenated chamigrene (figure 11) nor 4S, 5R-antillatoxin A (figure 12) gave promising results with purified mammalian sAC, leaving laurencione monoacetate as the best candidate for a novel sAC activator.

To determine the efficacy of the drugs in live cells, a human embryonic kidney cell line over-expressing rat sAC_t (4-4 HEK cells) was obtained from Lonny Levin and Jochen Buck's laboratory at the Weill Cornell Medical College. Over-expression of the rat sAC_t protein was expected to emphasize the stimulatory effects of laurencione monoacetate on cAMP production in the cells. Wild-type HEK cells were not available for culture to compare against the 4-4 HEK cells. Wild-type mouse embryonic fibroblast cells (MEF WT), and a sAC gene-knockout variant of the MEF cells (MEF sAC-KO) were cultured instead.

Cellular cAMP accumulation assays with the 4-4 HEK cells were optimized with drugs known to modulate the activities of mammalian adenylyl cyclases (figure

13). Isobutylmethylxanthine (IBMX) was included in all experimental conditions in order to prevent the degradation of cAMP by cellular phosphodiesterases. Experimental conditions included the sAC-specific inhibitor KH7, the tmAC inhibitor P-site, and the tmAC activator forskolin (FSK).

Data represents three replicates per experimental condition (figure 13). Basal cAMP production was inhibited by 93% with 30 μ M KH7, confirming the activity of rat sAC_t in the 4-4 HEK cells. This response was not significant with the given number of replicates. Treatment with 50 μ M P-site did not yield significant changes in cAMP production, suggesting that the cAMP produced by 4-4 HEK cells under control conditions was not due to tmAC activity. However, the presence and activity of tmAC was apparent with 100 μ M forskolin, which stimulated cAMP production ~10-fold relative to the control ($p < 0.0001$, one-way ANOVA and Tukey post-hoc test). An experimental condition combining both P-site and forskolin was not tested because of the limited number of wells available per assay plate. This experiment was done to optimize cAMP accumulation assay protocols in the live cells.

The dose response for 4-4 HEK cells treated with laurencione monoacetate showed no signs of stimulated cAMP production (figure 14). Cellular assays did not include HCO_3^- treatments because the cells were grown in media that already contained 0.15% HCO_3^- , and cultured in a 5% CO_2 incubator. The drug could have been metabolized as soon as it entered the cell, or exported before it could interact with its target protein. Assays combining the effects of laurencione monoacetate and KH7 in 4-4 HEK cells gave unexpected results (figure 15). Cells treated with either 30 μ M KH7, or 0.05 μ M laurencione monoacetate alone resulted in statistically

insignificant decreases in cAMP production relative to the control. However, a combined treatment with both 30 μ M KH7 and 0.05 μ M laurencione monoacetate resulted in a 90% stimulation of cAMP production ($p=0.0033$, one-way ANOVA and Tukey post-hoc test). The effects of laurencione monoacetate in 4-4 HEK cells were only observed when sAC was inhibited by KH7, raising questions about its role and target in the cAMP signaling pathway. Experiments with purified rat sAC_t suggested that the drug was capable of stimulating sAC in the absence of HCO_3^- . Yet these experiments with laurencione monoacetate in 4-4 HEK cells suggest that it may target another aspect of the cAMP signaling pathway that is regulated by sAC activity.

Changes in cAMP signals within subcellular microdomains might not be detectable with the whole cell lysates. Therefore cellular cAMP accumulation assays were complemented by an alternative method for visualizing changes in the cAMP signaling pathway. Protein kinase A (PKA) is a major downstream effector of cAMP that phosphorylates a number of proteins in the nucleus and cytoplasm in order to regulate transcription and mediate physiological responses (reviewed in Gancedo, 2013). Subtle changes in cAMP concentrations within a signaling domain get amplified by PKA, which could result in unique patterns of protein phosphorylation. Effects on the cAMP signaling pathway by laurencione monoacetate would also likely affect PKA activity in the cells. Protein phosphorylation was visualized in Western blots with a phospho-(Ser/Thr) PKA substrate antibody, which recognizes the phosphorylated target sequences of the PKA enzyme.

In each Western blot, a control DMSO treatment established the basal protein phosphorylation state for each cell line (4-4 HEK cells, MEF WT cells, and MEF

sAC-KO cells). It is important to note that phosphorylation patterns between the MEF WT and MEF sAC-KO controls matched, suggesting that the sAC deletion did not affect basal PKA activity in the KO cells. Cells were first treated with 100 μ M forskolin, which was expected to affect PKA activity by activating tmAC (figure 16). Previous experiments confirmed that forskolin caused cAMP concentrations to increase $\sim$ 10-fold in the 4-4 HEK cells (figure 13). However, Western blots only revealed the disappearance of two bands at $\sim$ 50 KDa, suggesting that the HEK cells actually lost phosphorylation with forskolin treatment. On the other hand, changes in PKA activity by forskolin were more apparent in the mice cells. The sizes of the heavier proteins above 100 KDa were similarly affected in the MEF WT and MEF sAC-KO cells. Three bands above 100 KDa in the control lanes were replaced by two bands of different sizes (one above 230 KDa, and the other just above 100 KDa) in the forskolin treatments. Additionally, the MEF sAC-KO cells gained a faint band at $\sim$ 70 KDa that was not observed in either the control treatments or the WT cells.

The effect of 50 μ M laurencione monoacetate on PKA phosphorylation in each cell line was also determined by Western blot (figure 17). Notably, a unique band signal at $\sim$ 37 KDa was gained in 4-4 HEK cells following treatment, providing evidence that laurencione monoacetate can affect PKA activity in these cells. This response also suggests that laurencione monoacetate affected PKA by means distinct from forskolin, since its effect on protein phosphorylation in the 4-4 HEK cells was different. Changes in protein phosphorylation by laurencione monoacetate were much more subtle in the mice cells. In the MEF WT cells, the 30 KDa band became slightly more intense with treatment. In the MEF sAC-KO cells, the 250 KDa band became

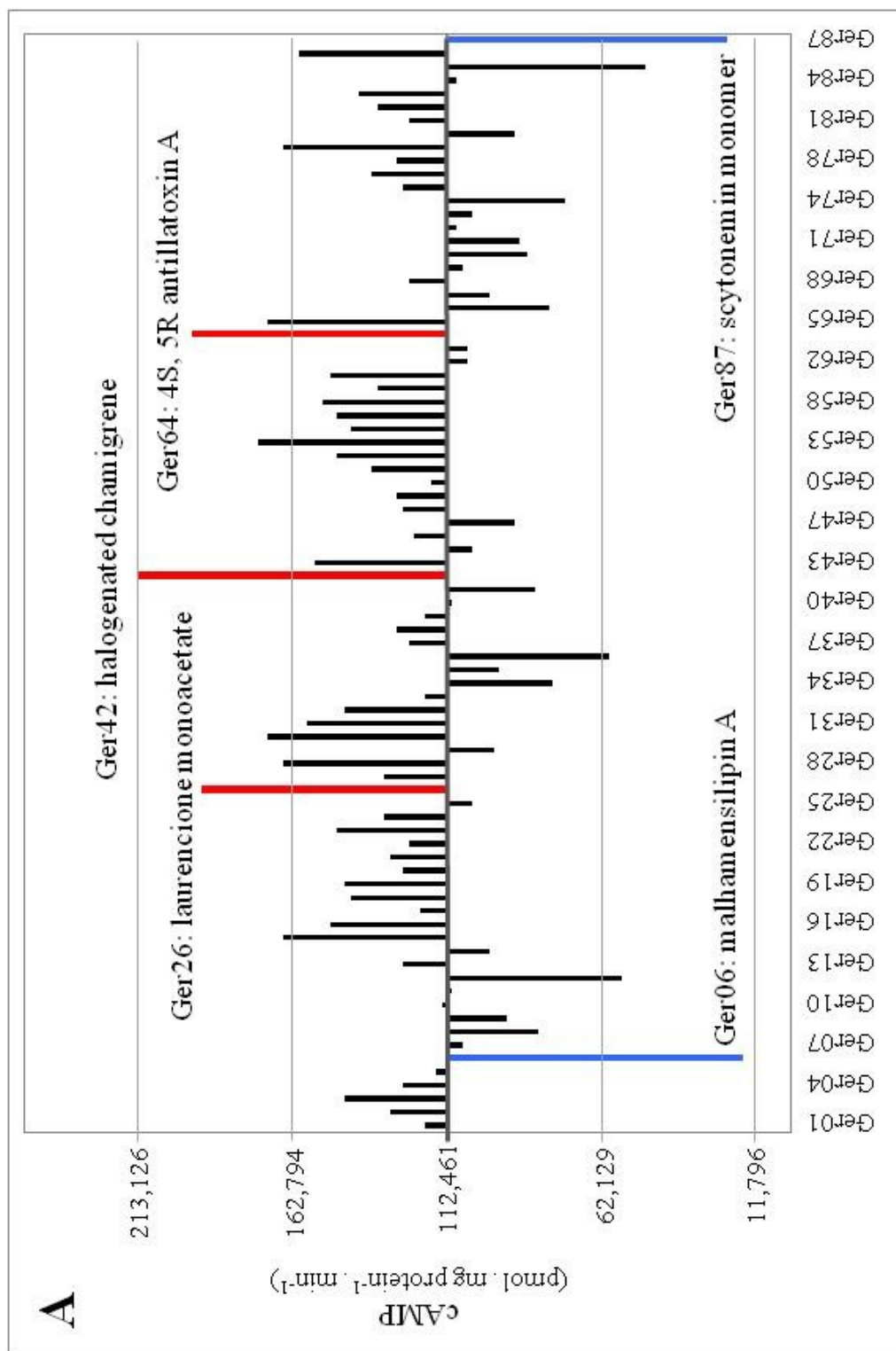
slightly fainter. Additional replicates would be necessary in order to draw relevant conclusions about the effects of the compound in the mice cells.

A Western blot of cells treated with 4S, 5R-antillatoxin A was also used to determine its effects on intracellular phosphorylation (figure 18). Though purified rat sAC_t was insensitive to 4S, 5R-antillatoxin A, assays with the coral tissue homogenates suggested that the compound had potential to affect the cAMP signaling pathway with HCO₃⁻ (figure 7). Treatments did not significantly affect phosphorylation patterns in the 4-4 HEK cells, though the band at ~27 KDa became more intense. A common loss of phosphorylation in the heavier proteins above 100 KDa was observed in the mice cells, though all of the bands in these lanes seemed fainter, suggesting a discrepancy in the loading concentrations. Additional replicates would be necessary in order to draw relevant conclusions about the effects of 4S, 5R-antillatoxin A on PKA activity in mammalian cells.

While forskolin affected PKA activity in all three cell lines, the other drugs elicited more subtle responses. Laurencione monoacetate did not seem to significantly affect PKA activity in the MEF cells, but caused a band at ~37 KDa to appear in the 4-4 HEK cells. 4S, 5R-antillatoxin A might have affected the intensities of the bands, but did not cause any significant changes in PKA phosphorylation patterns. These varied responses suggest that each drug was capable of affecting PKA activity by independent means, whether or not they directly affected cAMP production.

FIGURES

Figure 1: Cyclic AMP production in *P. damicornis* tissue homogenates in response to A) compounds from plate #1 (N=1) or B) compounds from plate #2 of the Gerwick laboratory's pure compound library (N=1). Data points represent single replicates of coral tissue treated with 10 µg/mL of each pure compound. Data is graphed relative to a control (two replicates of coral tissue treated with DMSO), which is represented by the thick bar in each graph. The two bars above and below this basal activity represent $\pm$ one and two standard deviations from the control. Red columns indicate strong activation of cAMP production, while blue columns indicate strong inhibition of cAMP production.



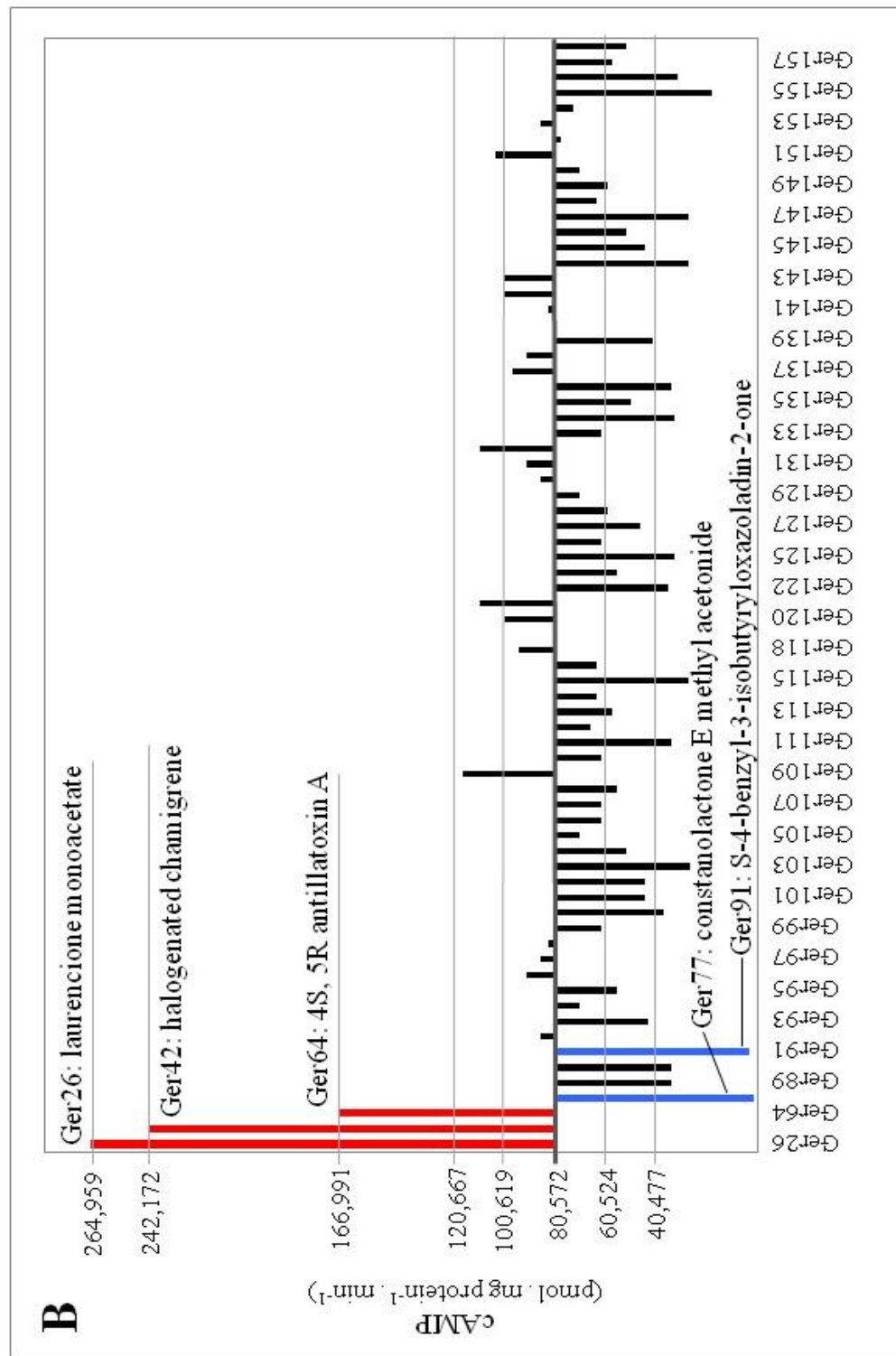
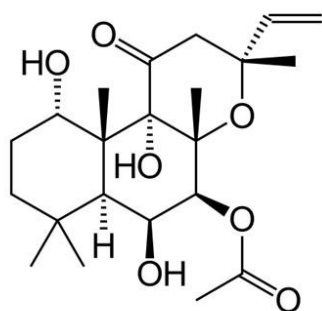


Figure 1: Cyclic AMP production in *P. daniicornis* tissue homogenates in response to A) compounds from plate #1 (N=1) or B) compounds from plate #2 of the Gerwick laboratory's pure compound library (N=1). Continued.

A**Forskolin**

(Seamon et al., 1981)

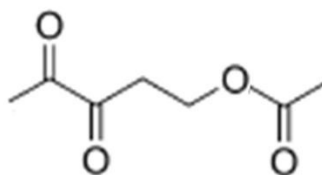
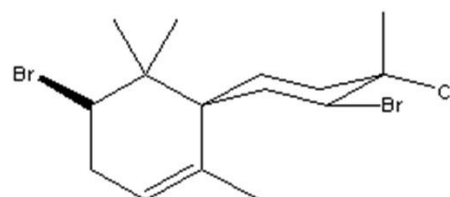
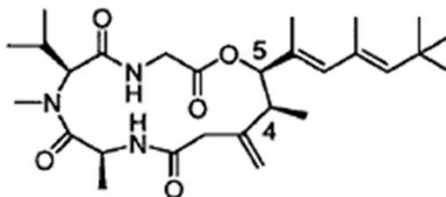
B**Laurencione monoacetate** $C_6H_8O_5 = 158.1519 \text{ g/mol}$ **Halogenated chamigrene** $C_{15}H_{23}Br_2Cl = 398.6041 \text{ g/mol}$ **4S, 5R-antillatoxin A** $C_{28}H_{45}N_3O_5 = 503.674 \text{ g/mol}$

Figure 2: Chemical structure of A) forskolin, an activator of mammalian tmAC, and the structures of B) the potential activators of cAMP production in *P. damicornis* coral tissue homogenates. The chemical structures for the potential activators were obtained from the Gerwick laboratory's chemical library database.

Table 1: Summary of biological activities for forskolin and the potential activators of cAMP production in *P. damicornis* tissue homogenates. The candidates for adenylyl cyclase activators identified in figure 1 (highlighted in red) were further investigated.

Characterized activators of adenylyl cyclases			
Compound	Citation	Efficacy	Notes
Forskolin	Seamon et al., 1981 Chen et al., 2000 Pinto et al., 2008	EC ₅₀ =10 μ M	General tmAC activator in mammals. Does not affect sAC activity. Forskolin analogs were designed to activate tmAC isoforms with greater selectivity.
Potential activators of cAMP in <i>P. damicornis</i>			
Compound	Organism	Molecular weight (g/mol)	Notes
Laurencione monoacetate	<i>Laurencia spectabilis</i>	158.15	Structural similarity to acyl-homoserine lactones (AHL). Potential inhibition of quorum sensing and inflammatory responses (Gerwick et al. 2012).
Halogenated chamigrene	<i>Laurencia</i> sp.	398.6	Similar derivatives of chamigrenes isolated from <i>Laurencia</i> show potential antibacterial properties (Bansemir et al. 2004).
4S, 5R-antillatoxin A	<i>Moorea producens</i>	503.67	Potent ichthyotoxin that activates voltage-gated Na ⁺ channels (Li et al. 2001). (4R, 5R)—stereoisomer has a 25-fold more potent ichthyotoxic effect (Li et al. 2004).

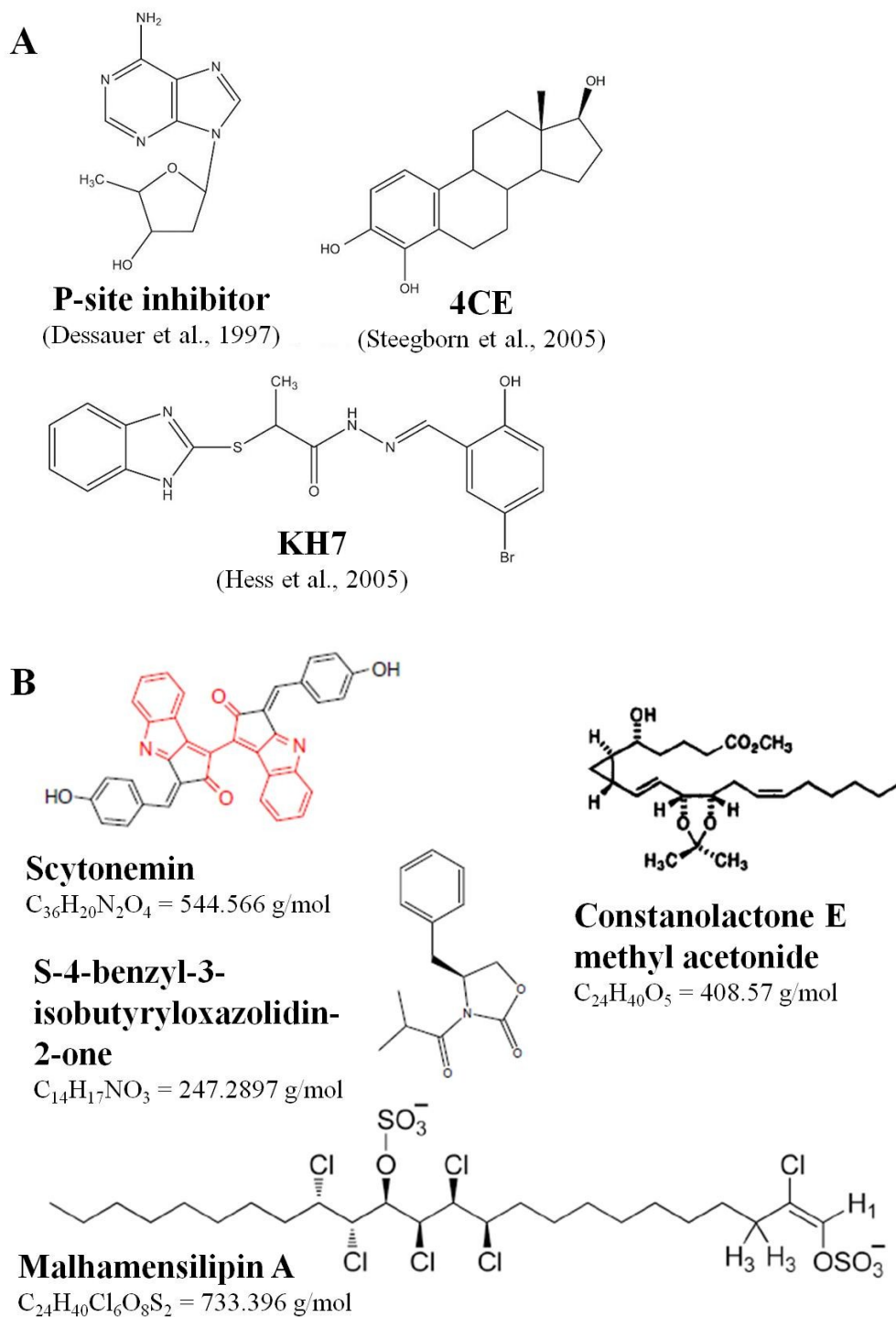


Figure 3: Chemical structures of A) the known inhibitors of adenylyl cyclases, and B) the potential inhibitors of cAMP production in *P. damicornis* tissue homogenates. The chemical structures for the potential inhibitors were obtained from the Gerwick laboratory's chemical library database.

Table 2: Summary of biological activities for characterized inhibitors of adenylyl cyclases, and for potential inhibitors of cAMP production in *P. damicornis* tissue homogenates. The candidates for adenylyl cyclase inhibitors identified in figure 1 (highlighted in blue) were further investigated.

Characterized inhibitors of adenylyl cyclases			
Compound	Citation	Efficacy	Notes
P-site Inhibitor	Dessauer and Gilman, 1997		Inhibit most tmAC isoforms by binding to the active site. Stabilize enzyme-product complex to prevent product release.
4CE	Steebom et al., 2005 Schlicker et al., 2008	IC ₅₀ = 2 µM	Non-specific inhibitor of adenylyl cyclases. Binds outside of the active site. Chelates Mg ²⁺ to inhibit AC activity. Analogs developed that preferentially inhibit sAC.
KH7	Hess et al., 2005	IC ₅₀ = 10 µM	sAC-specific inhibitor. Inert towards tmAC activity.
Potential inhibitors of cAMP in <i>P. damicornis</i>			
Compound	Organism	Molecular weight (g/mol)	Notes
Scytonemin	<i>Tolypothrix distorta</i>	544.57	Sunscreen molecule, absorbs UV-radiation (Jones et al., 2011).
S-4-benzyl-3-isobutyryloxazolidin-2-one	<i>Moorea producens</i>	247.29	
Constanolactone E methyl acetone	<i>Constantinea simplex</i>	408.57	
Malhamensilipin A	<i>Poteroochromonas malhamensis</i>	733.4	Antimicrobial compound, inhibitor of pp60 protein Tyrosine kinase (Chen et al., 1994).

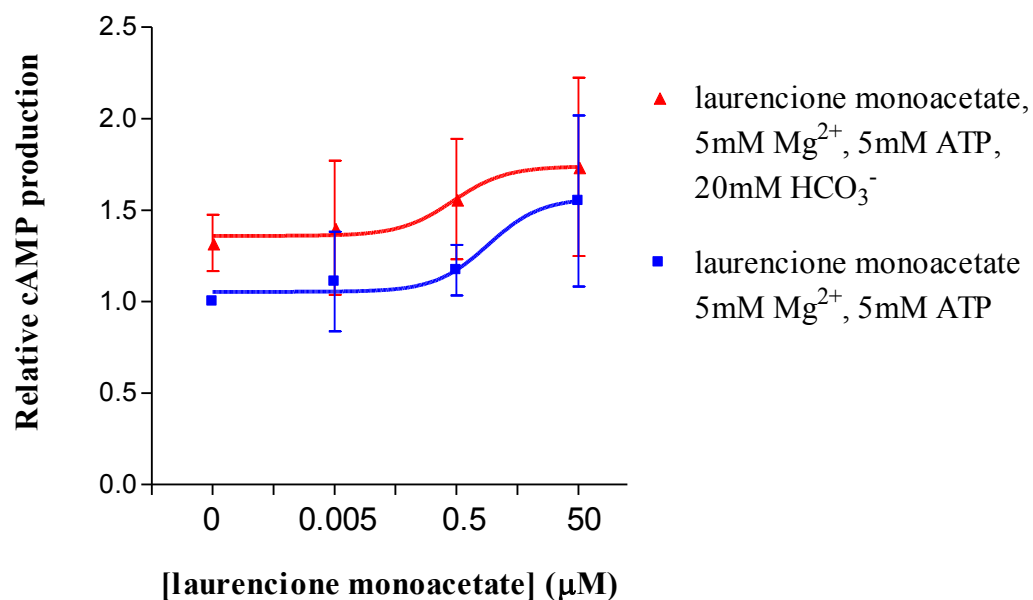


Figure 4: Dose response curve of laurencione monoacetate on cAMP production in *P. damicornis* tissue homogenates (N=4). Lines were drawn with a sigmoidal curve fit model. Error bars indicate SEM.

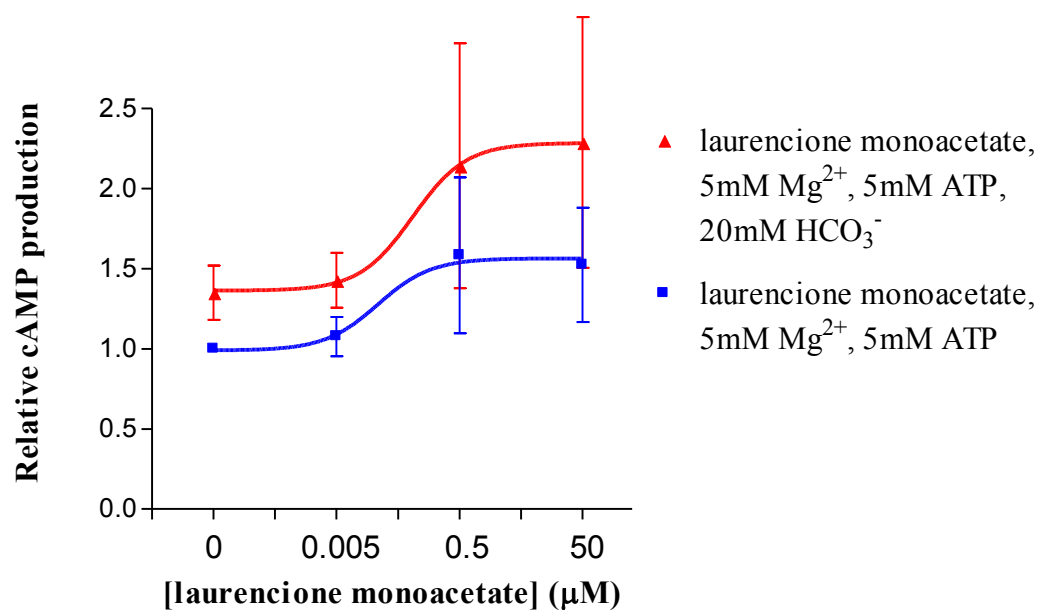


Figure 5: Dose response curve of laurencione monoacetate on cAMP production in *A. yongei* tissue homogenates (N=5). Lines were drawn with a sigmoidal curve fit model. Error bars indicate SEM.

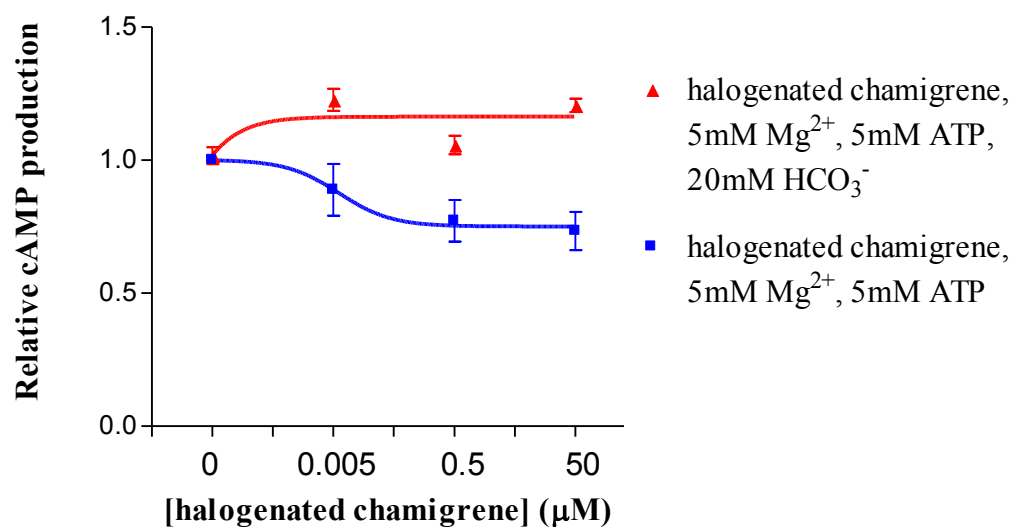


Figure 6: Dose response curve of halogenated chamigrene on cAMP production in *A. yongei* tissue homogenates (N=2). Lines were drawn with a sigmoidal curve fit model. Error bars indicate SEM.

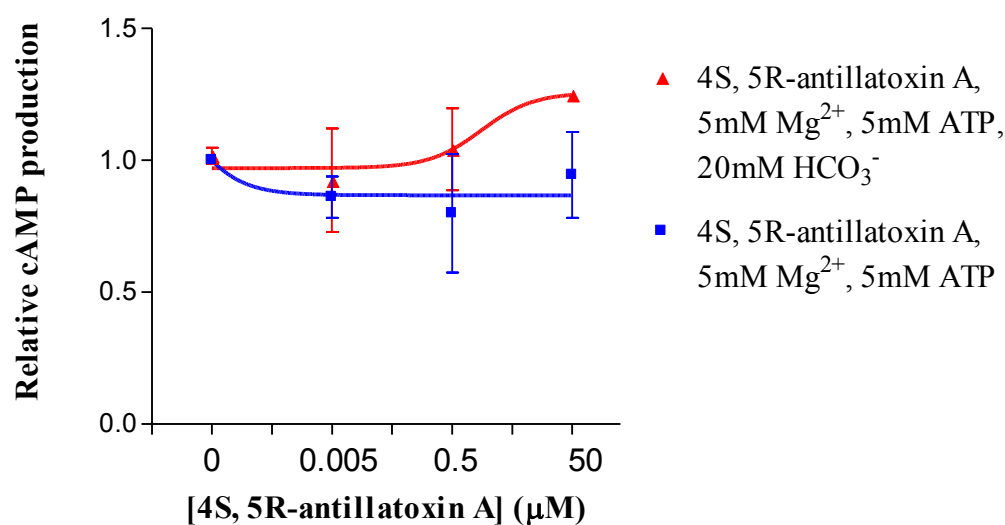


Figure 7: Relative dose response curve of 4S, 5R-antillatoxin A on cAMP production in *A. yongei* tissue homogenates (N=2). Lines were drawn with a sigmoidal curve fit model. Error bars indicate SEM.

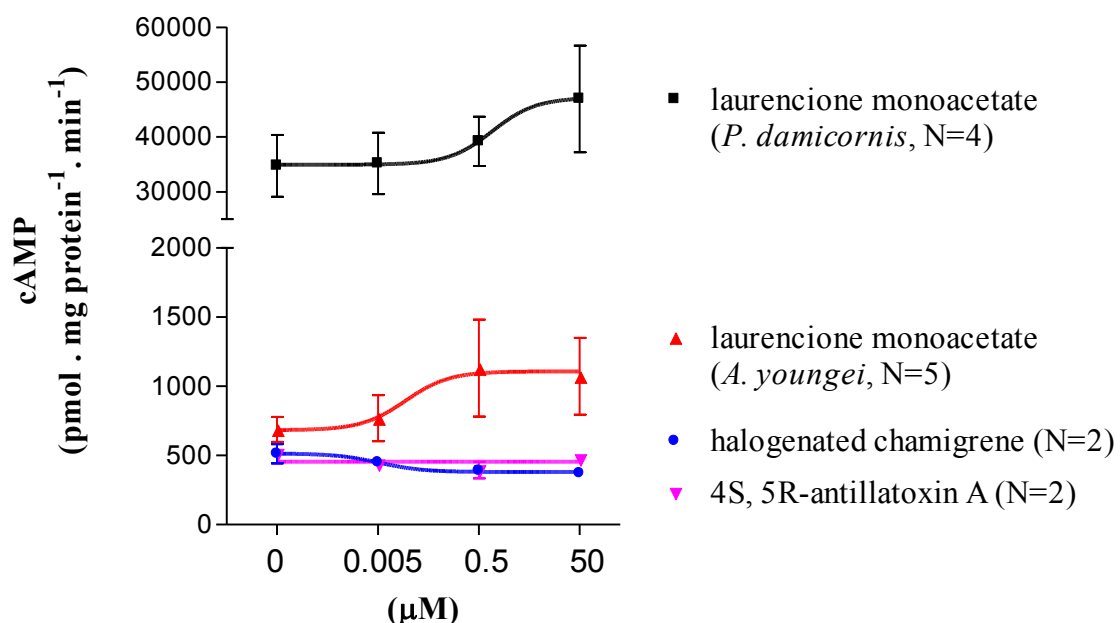


Figure 8: Dose response effects of laurencione monoacetate, halogenated chamigrene, and 4S, 5R-antillatoxin A on coral tissue homogenates. Lines were drawn with sigmoidal curve fit models. Error bars indicate SEM.

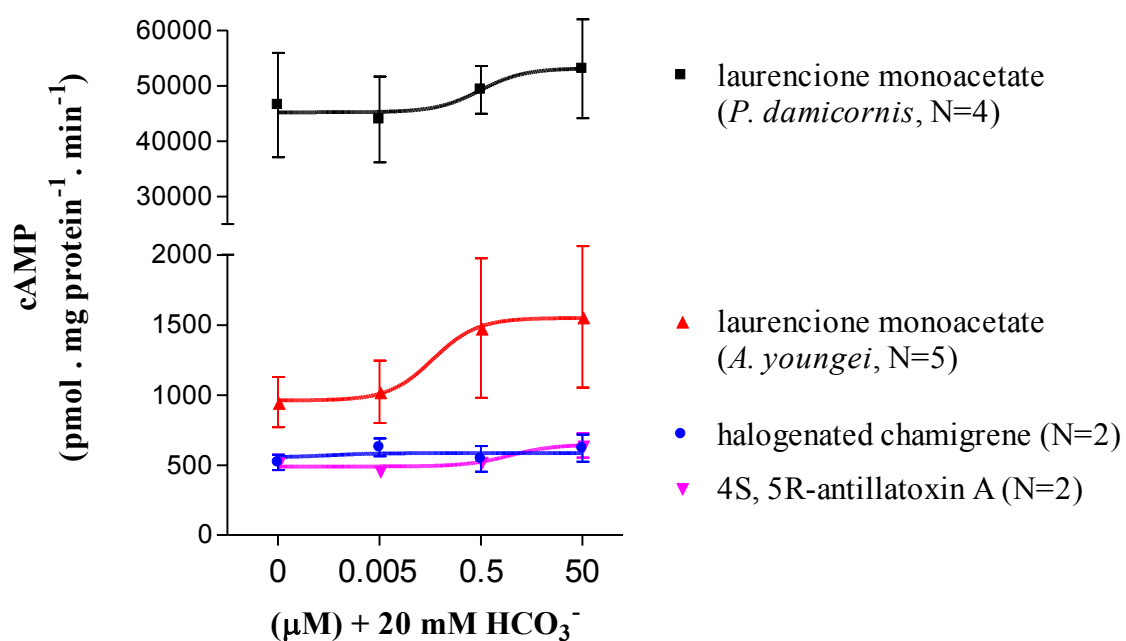


Figure 9: Dose response effects of laurencione monoacetate, halogenated chamigrene, and 4S, 5R-antillatoxin A with 20 mM HCO₃⁻ on coral tissue homogenates. Lines were drawn with sigmoidal curve fit models. Error bars indicate SEM.

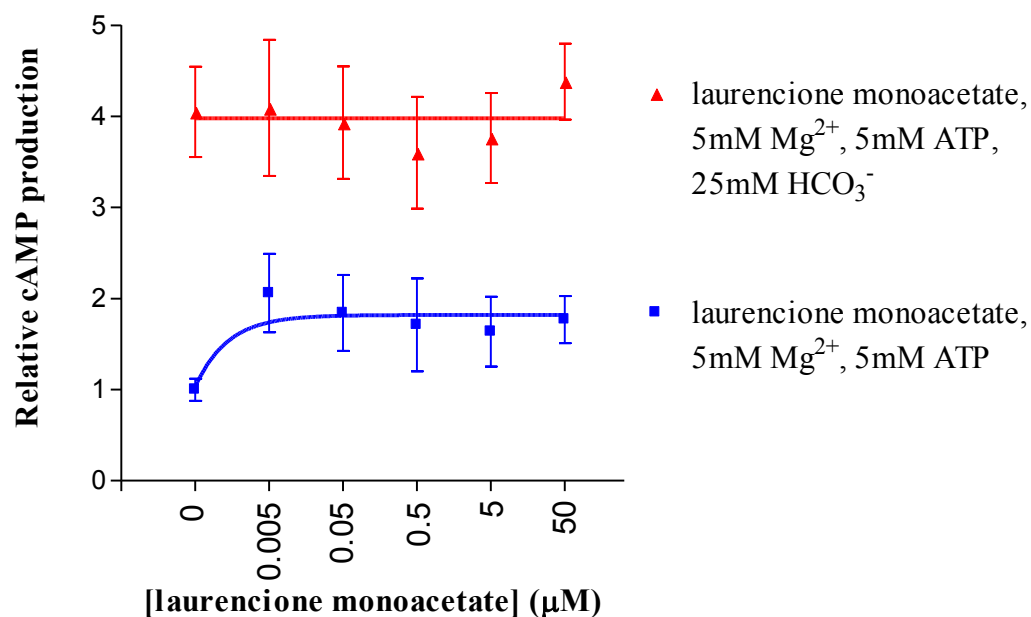


Figure 10: Relative dose response curve of laurencione monoacetate on cAMP production with purified rat sAC_t (N=15, from 6 different experiments). Lines were drawn with a sigmoidal curve fit model. Error bars indicate SEM.

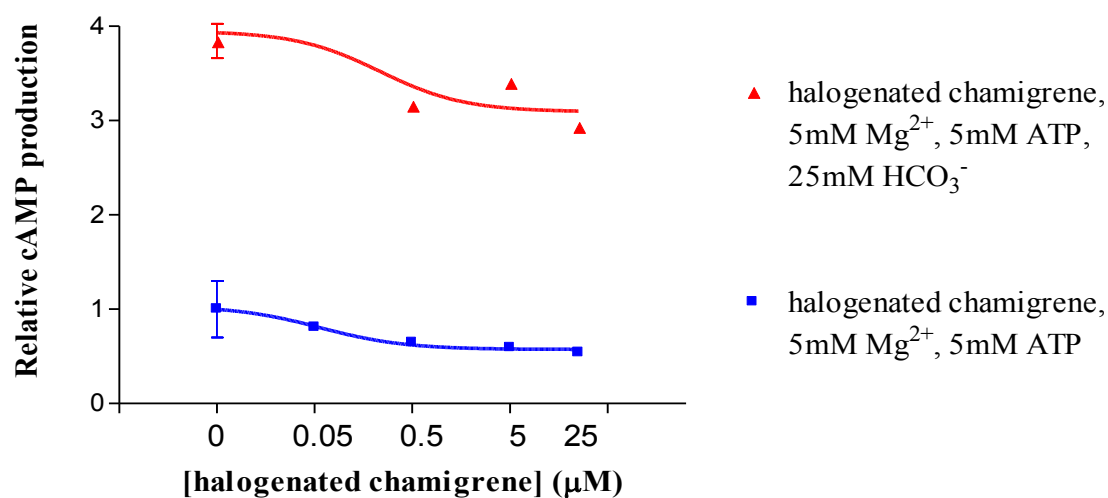


Figure 11: Dose response curve of the effect of halogenated chamigrene on cAMP production with purified rat sAC_t. DMSO and HCO_3^- controls are represented by N=2, from the same experiment. All other treatment conditions are represented by a single replicate. Lines were drawn with a sigmoidal curve fit model. Error bars indicate SEM.

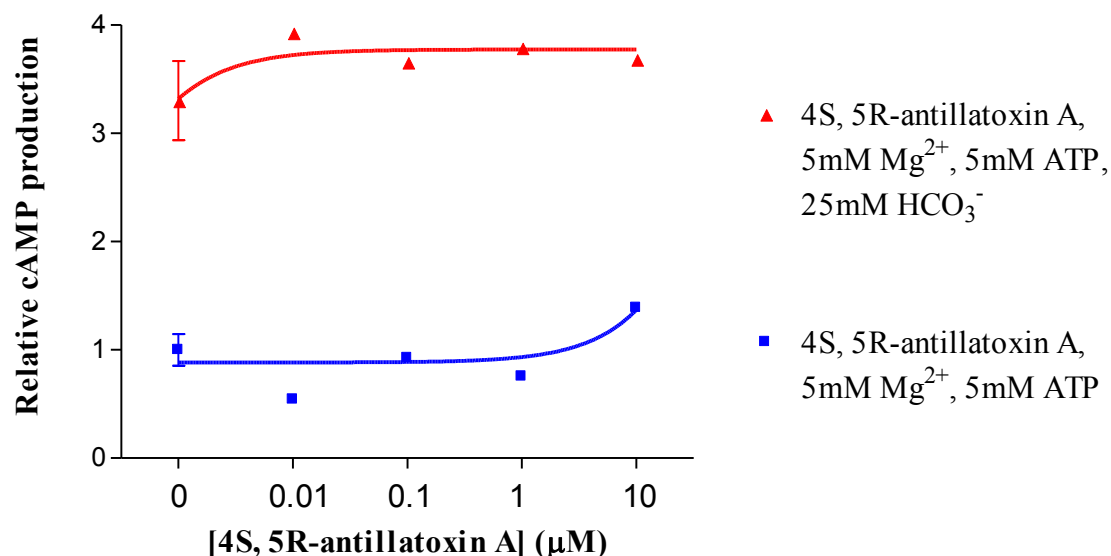


Figure 12: Dose response curve of the effect of 4S, 5R-antillatoxin A on cAMP production with purified rat sACT. DMSO and HCO_3^- controls are represented by N=4, from 2 separate experiments. All other treatment conditions are represented by a single replicate. Lines were drawn with a sigmoidal curve fit model. Error bars indicate SEM.

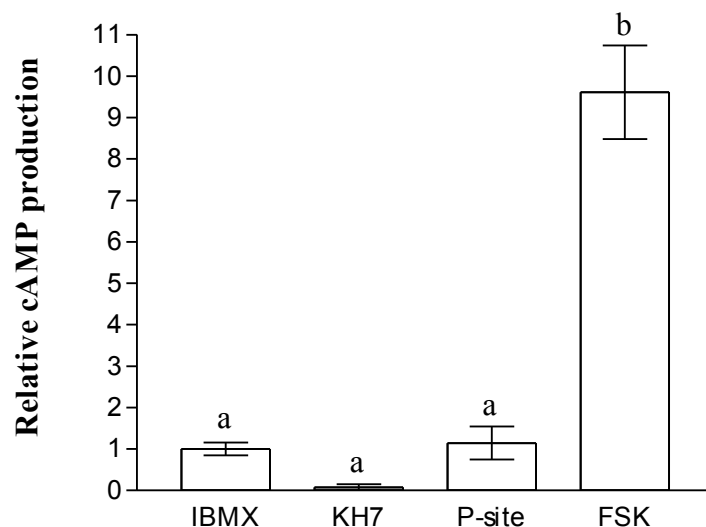


Figure 13: Relative cAMP production in 4-4 HEK cells treated with various pharmacological drugs (N=3, from a single experiment). Treatments: IBMX = 500 μM IBMX; KH7 = 30 μM KH7 + 500 μM IBMX; P-site = 50 μM P-site + 500 μM IBMX; FSK = 100 μM forskolin + 500 μM IBMX. Symbols indicate statistically different groups determined by one-way ANOVA and Tukey post-hoc test. Error bars indicate SEM.

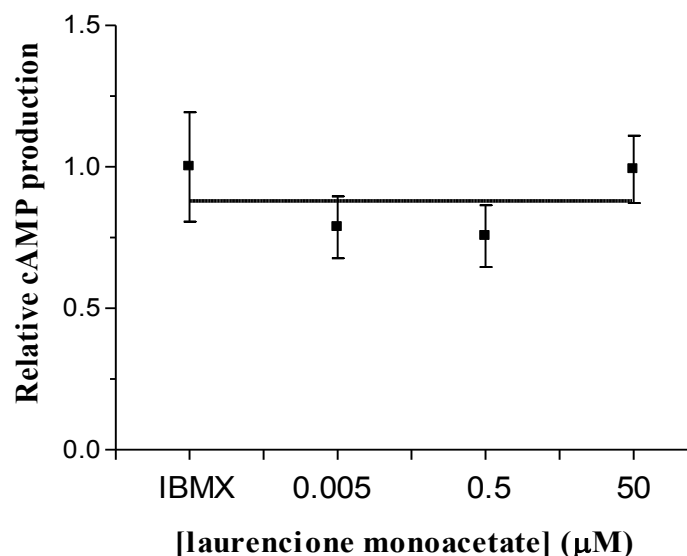


Figure 14: Relative dose response curve of laurencione monoacetate on cAMP production in 4-4 HEK cells (N=9, from 3 experiments conducted on 2 different days). All treatment conditions contained 500 μM IBMX. The line was drawn with a sigmoidal curve fit model. Error bars indicate SEM.

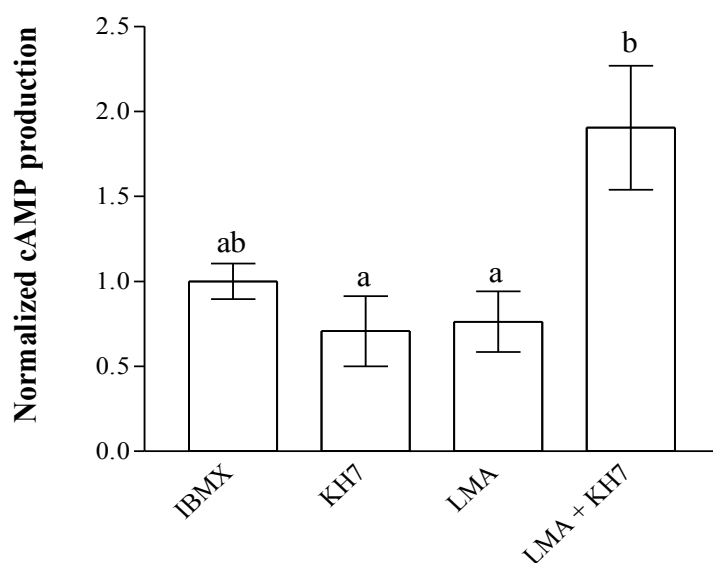


Figure 15: Relative cAMP production in 4-4 HEK cells treated with laurencione monoacetate and KH7 (N=9, from 3 experiments conducted on 3 different days). Treatments: IBMX = 500 μM IBMX; KH7 = 30 μM KH7 + 500 μM IBMX; LMA = 0.05 μM laurencione monoacetate + 500 μM IBMX; LMA + KH7 = 0.05 μM laurencione monoacetate + 30 μM KH7 + 500 μM IBMX. Symbols indicate statistically different groups determined by one-way ANOVA and Tukey post-hoc test. Error bars indicate SEM.

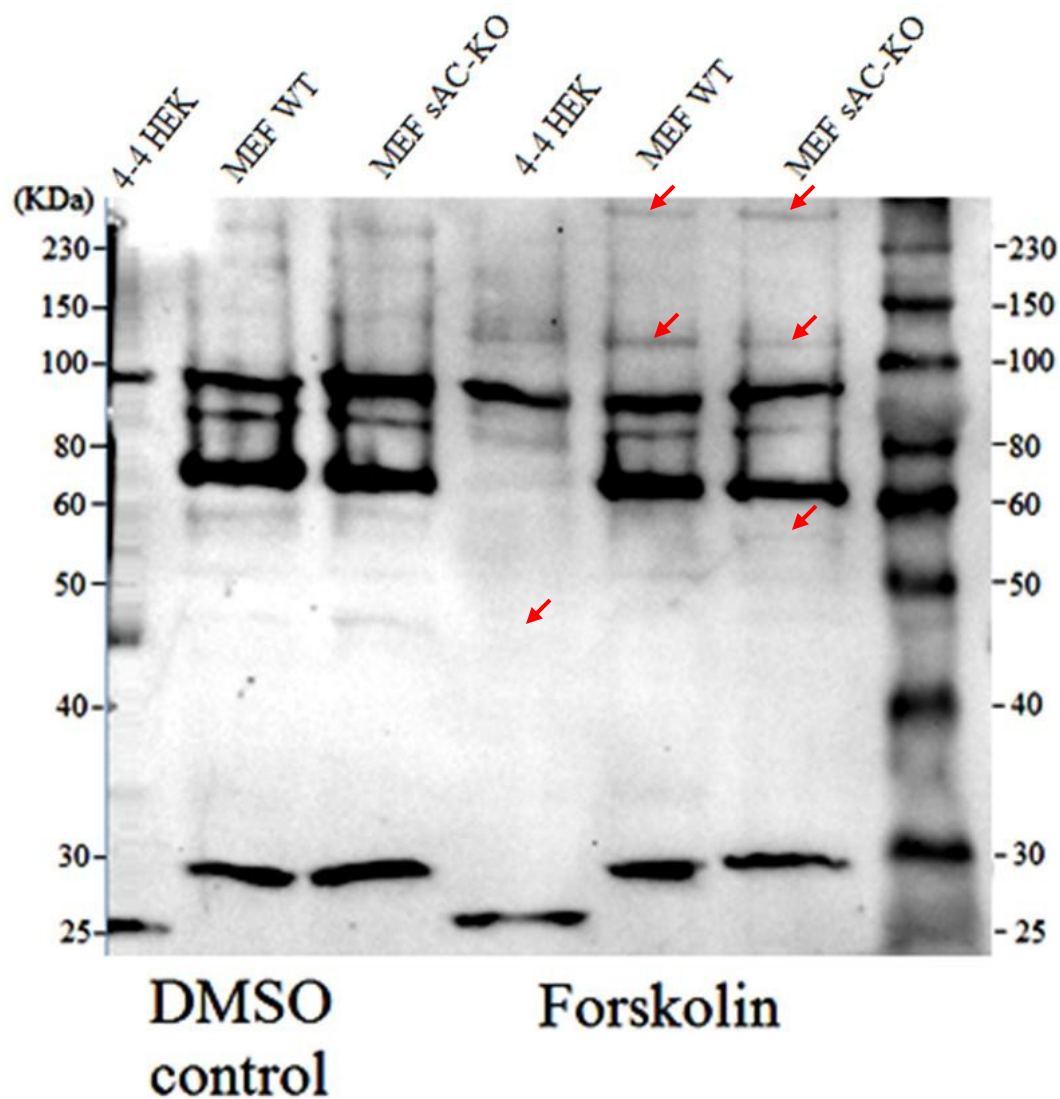


Figure 16: Western blot of PKA phosphorylation patterns in 4-4 HEK cells; MEF WT cells; and MEF KO cells treated with forskolin (N=2). Each lane was loaded with 5 μ g of protein from the corresponding cell line. Treatment conditions included a DMSO control, and 30 μ M forskolin. Arrows indicate changes in protein phosphorylation by PKA (relative to the DMSO control) following treatment.

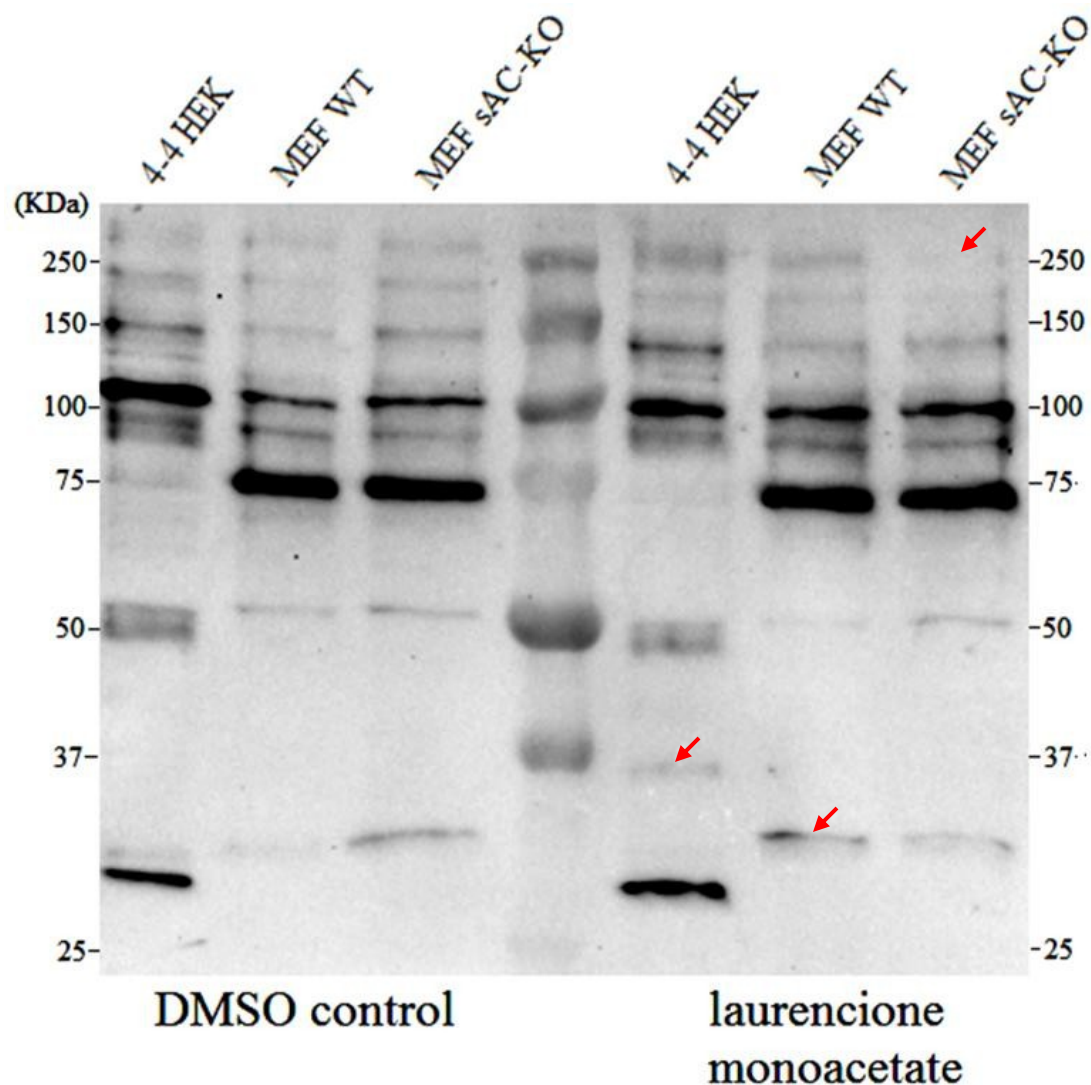


Figure 17: Western blot of PKA phosphorylation patterns in 4-4 HEK cells; MEF WT cells; and MEF KO cells treated with laurencione monoacetate (N=1). Each lane was loaded with 2.23 μg of protein from the corresponding cell line. Treatment conditions include a DMSO control, and 50 μM laurencione monoacetate. Arrows indicate changes in protein phosphorylation by PKA (relative to the DMSO control) following treatment.

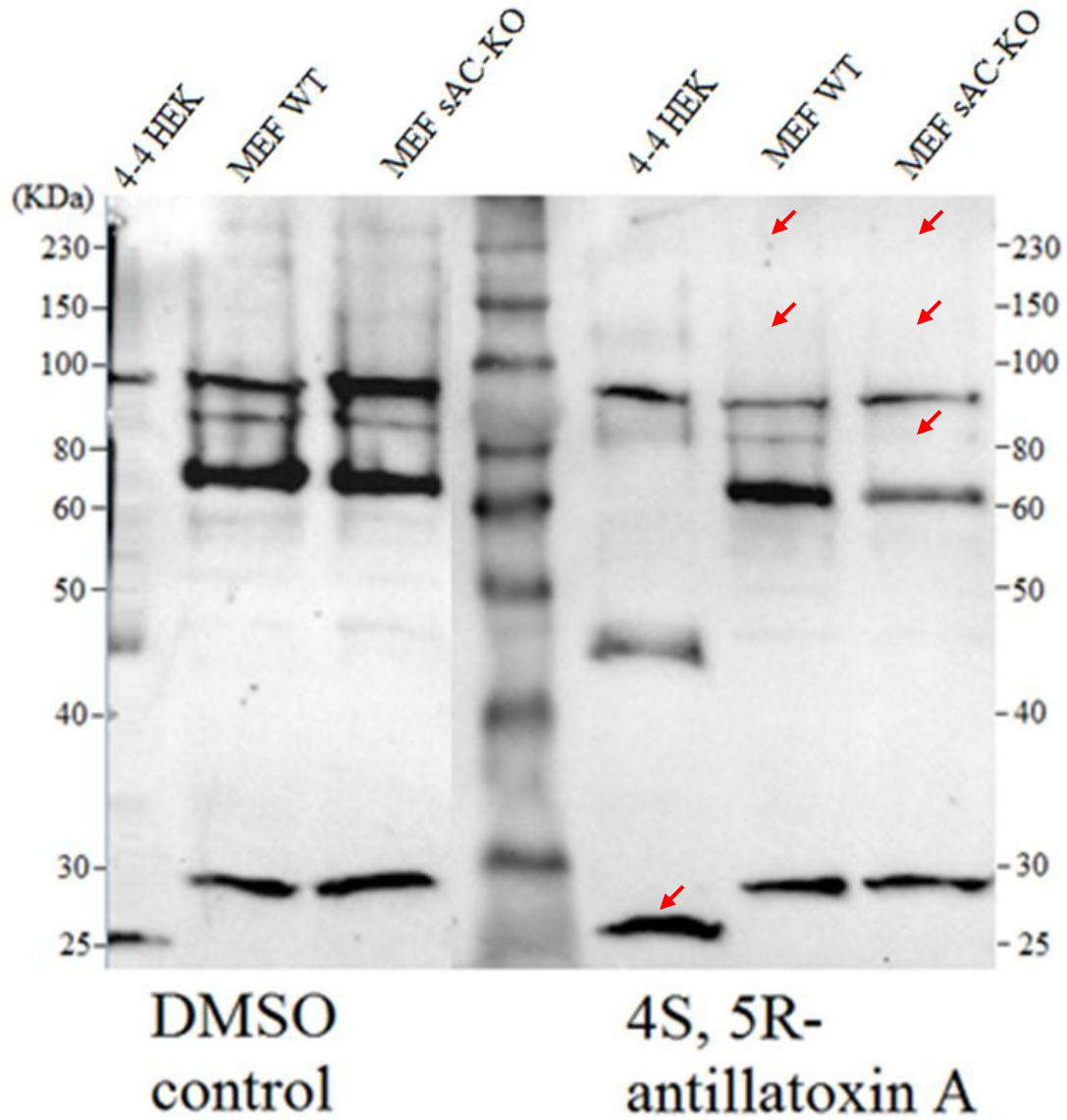


Figure 18: Western blot of PKA phosphorylation patterns in 4-4 HEK cells; MEF WT cells; and MEF KO cells treated with 4S, 5R-antillatoxin A (N=1). Each lane was loaded with 5 μ g of protein from the corresponding cell line. Treatment conditions include a DMSO control, and 50 μ M 4S, 5R-antillatoxin A. Arrows indicate changes in protein phosphorylation by PKA (relative to the DMSO control) following treatment.

DISCUSSION

A collection of pure marine natural products and their synthetic derivatives was obtained from the Gerwick laboratory at the Scripps Institution of Oceanography. Their effects on cAMP production were screened with tissue homogenates from the coral *Pocillopora damicornis*. This is the first time that coral tissues were used to identify drugs capable of affecting cAMP production. Corals were chosen as a study subject because of their unusually high adenylyl cyclase activity (Barott et al., 2013), making them excellent tools for identifying drugs that target adenylyl cyclases. One of the goals of this study was to identify an activator of sAC, which is insensitive to forskolin. As a physiological HCO_3^- -sensor, sAC may mediate $\text{CO}_2/\text{HCO}_3^-$ -dependent processes in corals such as photosynthesis and light-enhanced calcification. Circadian fluctuations in coral cAMP concentrations suggest that these diurnal processes may be regulated by cAMP (Barott et al., 2013). Furthermore, cAMP production in coral tissue homogenates was significantly inhibited by 50 μM KH7, demonstrating that sAC activity is relevant to coral physiology (Barott et al., 2013). Compounds that simulate adenylyl cyclase activity in corals could be activators of sAC.

The cAMP assays with coral tissue homogenates were used to narrow down the candidates for novel stimulators of cAMP production to just three compounds. High variability in cAMP produced by the DMSO controls meant that repeated experiments were necessary to confirm the effects of these drugs in corals. The variability in coral cAMP production could have been due to the heterogeneous nature of the coral tissue homogenates. Since it was unclear what proteins the drugs were affecting in the homogenized tissues, the effects of the drugs were also tested with

purified rat sAC_t protein. Follow up experiments revealed that halogenated chamigrene did not stimulate cAMP production in either coral tissue homogenates or in purified rat sAC_t. Thus research focused on characterizing the effects of the other two drugs instead.

Coral tissue homogenates treated with both 50 μ M 4S, 5R-antillatoxin A and 20 mM HCO₃⁻ produced ~25% more cAMP than the control. A HCO₃⁻-dependent response suggested the involvement of sAC activity, however the activity of purified rat sAC_t protein was insensitive to the drug. It is possible that these distinct responses were due to structural differences between the coral and rat sAC proteins, especially since rat sAC_t was a truncated isoform containing only the catalytic domains. A Western blot of mammalian cells treated with 4S, 5R-antillatoxin A did not show any major changes in PKA activity relative to the control. The intensities of a few bands may have been affected; however replicate experiments would be necessary to draw any relevant conclusions. The stereoisomers of antillatoxin A have been characterized as potent activators of voltage-gated Na⁺ channels (Li et al., 2001). Its comparatively weak effect in these experiments suggests that apparent stimulation of cAMP production by 4S, 5R-antillatoxin A was likely coincidental.

The effects of laurencione monoacetate were tested on both *Pocillopora damicornis* and *Acropora yongei* coral tissue homogenates. Though distinct rates of cAMP production have been established in the two species (Barott et al., 2013), the differences in basal rates measured for this study were even greater than expected. The basal rate of cAMP production from *A. yongei* was ~700 pmol · mg_{prot}⁻¹ · min⁻¹, which was ~25 times lower than reported in Barott et al., 2013. This disparity could be

due to differences in the individual corals sampled, or in tissue sampling protocols. For example, the *A. yongei* coral fragments used in this study could have been sampled during conditions in which cAMP production was not ideal. On the other hand, the basal rate of cAMP production in *P. damicornis* was comparable to the reported value, since the samples were homogenized from the same specimens. Treatments with 50 μ M laurencione monoacetate elicited a similar 60% stimulation of cAMP production in both species, with a low EC_{50} of ~ 25 nM—1 μ M. Though this response was not statistically significant in either species, laurencione monoacetate was considered the best candidate for an activator of cAMP production because of its greater stimulatory effect in both coral species.

Structural studies describing the catalytic mechanism of the cyanobacterial sAC homolog CyaC, provide insight on how ions influence sAC activity. Ca^{2+} binds to the catalytic domain and increases substrate affinity by coordinating the phosphate groups of ATP (Steegborn et al., 2005 B). On the other hand, HCO_3^- mediates sAC activity by relieving substrate inhibition, and by stimulating the substrate turnover rate (Litvin et al., 2003; Steegborn et al., 2005 B). In the absence of HCO_3^- , high concentrations of ATP- Mg^{2+} can actually lead to decreased sAC activity (Litvin et al., 2003). Though the mechanism of this inhibition is still unclear, it is possibly due to a natural rate-limiting step in either product formation or product release. HCO_3^- facilitates both processes by inducing a conformational change and recruiting the catalytic Mg^{2+} ion to the active site (Steegborn et al., 2005 B). In other words, HCO_3^- increases the rate of substrate turnover (V_{max}), while Ca^{2+} increases the substrate affinity (K_m) of the sAC enzyme (Litvin et al., 2003; Steegborn et al., 2005 B).

Dose response effects of laurencione monoacetate with purified rat sAC_t were consistent with its effects on coral tissue homogenates. The basal activity of rat sAC_t was stimulated ~80% with 50 μ M treatment. The EC₅₀ of this dose response was lower than 5 nM, suggesting that the drug had an extremely high affinity for the rat sAC_t enzyme. However, the stimulatory effect of laurencione monoacetate on purified rat sAC_t was not evident with 25 mM HCO₃⁻. This lack of response with HCO₃⁻ undermines its potential as a pharmacological tool for use *in vivo*, since HCO₃⁻ is a physiological buffer present in all cells. It is possible that the activity of rat sAC_t was maximally activated by HCO₃⁻, preventing it from being further stimulated with laurencione monoacetate treatment. The effects of HCO₃⁻ and laurencione monoacetate on the truncated rat sAC isoform suggest that both compounds bind within or near the catalytic domain. There is the potential that both compounds share the same sAC binding domain, due to structural similarities between the acetate group of laurencione monoacetate and HCO₃⁻. Competition for the same binding site could explain why their effects on sAC were not synergistic. However, evidence suggests that the conformational changes due to HCO₃⁻ are transient, and that interactions between HCO₃⁻ and sAC are very brief (Steegborn et al., 2005 B).

Human embryonic kidney cells that over-expressed the rat sAC_t protein (4-4 HEK cells) were used to test the effect of laurencione monoacetate on live cells. In order for the drug to have an effect in cells, it would first need to be able to pass through the cellular membrane and avoid degradation from native enzymes. It would also need to remain in the cell and be able to reach its target. Laurencione monoacetate was likely able to enter the cells due to its small, hydrophobic structure. However,

treatment of the 4-4 HEK cells with laurencione monoacetate did not result in a detectable change in cAMP accumulation. It is important to note that cAMP concentrations were measured from the entire cellular lysate, which meant a loss in resolution with cAMP signals in subcellular microdomains. Even though the cells were over-expressing rat sAC_t, the distribution of sAC was probably not uniform throughout the entire cell. For example in COS7 cells, sAC was preferentially localized in the nucleus, mitochondria, and in the cytoplasm directly adjacent to the nucleus (Zippin et al., 2003). By measuring cAMP concentrations from the entire cell, the effects of laurencione monoacetate on sAC activity in subcellular compartments might get lost amid the background.

Surprising results from a different set of experiments with 4-4 HEK cells suggest the potential for cross-talk effects by laurencione monoacetate. Concentrations of cAMP in the 4-4 HEK cells were not significantly affected by either 30 μ M KH7 or 50 nM laurencione monoacetate treatments. The sAC-specific inhibitor KH7 decreased cAMP concentrations within the cells by ~30%, though this response was not statistically significant. A combined treatment with both drugs resulted in an unexpected 90% increase in accumulated cAMP. This combined response suggests that laurencione monoacetate could influence another aspect of cAMP production that is normally inhibited by sAC, though the exact mechanism by which KH7 inhibits sAC activity is still not characterized. Putative functions for sAC include sperm capacitation in rat testes (Hess et al., 2005), insulin secretion in pancreatic cells (Zippin et al., 2013), regulation of gene expression via PKA and CREB in the nucleus (Zippin et al., 2004), and the formation of mitotic spindles during cell division (Zippin

et al., 2003). It is also involved in acid/base sensing and regulation of fluid and ion transport in kidney cells (reviewed in Tresguerres et al., 2011). It is possible that laurencione monoacetate may affect another aspect of the cAMP signaling pathway that is regulated by downstream effectors of sAC such as PKA and Epac. Additional experiments would be necessary to confirm the hypothesis that cross-talk effects from laurencione monoacetate can raise cAMP concentrations in cells treated with the sAC inhibitor KH7. Characterization of a compound that can specifically affect sAC activity is challenging due to the complex nature of the cAMP signaling pathway.

An alternative method for visualizing changes in the cAMP signaling pathway was through detecting PKA activity in Western blots. Proteins from cellular lysates were separated by size with SDS-PAGE, then stained with an antibody that recognized the phosphorylated target sequence of the PKA enzyme. Pharmacological effects on cAMP concentrations within distinct signaling domains could produce unique patterns of protein phosphorylation. Basal phosphorylation states for each cell line (4-4 HEK, MEF WT and MEF sAC-KO) were determined by a control DMSO treatment. Notably, the patterns of protein phosphorylation between MEF WT and MEF sAC-KO cells were the same, suggesting that their basal PKA activity was not significantly affected by the sAC deletion. Evidence suggests that kidney and brain cells from sAC-KO mice may express a somatic isoform of sAC that is not affected by the deletion of the first few exons (Farrell et al., 2008). It is possible that the MEF sAC-KO cells used in these experiments may also express this somatic isoform of sAC, though this has not been confirmed.

Though no changes in cAMP concentration were detected in 4-4 HEK cells treated with laurencione monoacetate, changes in protein phosphorylation from the Western blot suggested that treatment could affect PKA activity. The appearance of a novel band at ~37 KDa provided evidence that laurencione monoacetate could affect protein phosphorylation in 4-4 HEK cells via PKA. On the other hand, no significant changes were observed in either the MEF WT or the MEF KO cells, aside from subtle differences in the band intensities. The fact that laurencione monoacetate stimulated the basal activity of purified rat sAC_t, coupled with its effect on protein phosphorylation in 4-4 HEK cells, suggests that laurencione monoacetate is capable of influencing cAMP signals in live cells. However, changes in PKA activity may also be due to factors not related to cAMP production. For example, phosphatases are enzymes that cleave the phosphate groups from phosphorylated proteins. In the mitochondria, phosphatase activity is the dominant factor regulating PKA phosphorylation, rather than cAMP concentrations in the mitochondrial matrix (Lefkimmatis et al., 2013). Furthermore, Western blot treatments did not include IBMX, so PDE activity could have degraded cAMP signals and limited the effects of the drugs on PKA activity. Though it was unclear if laurencione monoacetate actually stimulated cAMP production in the live cells, its effect on protein phosphorylation was distinct from forskolin. This difference in response suggests that the drugs affected PKA activity by discrete means.

The original laurencione natural product has been implicated as a regulator of quorum sensing, capable of inducing bioluminescence in the cyanobacteria *Vibrio harveyi* (Lowery et al., 2005). The monoacetate analog used for this study was also

capable of inhibiting NO production in macrophages exposed to bacterial lipopolysaccharides (Gerwick et al., 2012). This study demonstrated that laurencione monoacetate increased cAMP production in coral tissue homogenates, and was capable of stimulating basal rat sAC_t activity with high affinity. Bacterial quorum sensing systems have long been associated with cAMP signals and cAMP receptor proteins (CRP). In *Vibrio fischeri*, cAMP induced transcription of the luxR gene via CRP, which in turn induced transcription of the *lux* genes responsible for bioluminescence (Dunlap and Greenberg, 1988). In *Vibrio vulnificus*, cAMP signals were indicators of glucose availability, and were capable of modulating cellular metabolism and virulence gene expression (Kim et al., 2012). Cyclic AMP has also been demonstrated to affect nitric oxide production in mammalian cell systems. Exendin-4 is a potent glucagon-like peptide (GLP) receptor agonist that decreased NO production in LPS-stimulated macrophages via stimulation of tmAC (Chang et al., 2013). Activation of cAMP and PKA by Exendin-4 signaled the direct degradation of nitric oxide synthase, resulting in decreased NO production (Chang et al., 2013). In hepatocytes, inhibition of nitric oxide synthase occurred at a transcriptional level via cAMP and Epac (Zhang et al., 2013). All of the reported effects of laurencione monoacetate can be linked to the cAMP signaling pathway, reinforcing its proposed role as a stimulator of cAMP production and sAC activity.

This study demonstrates that the high adenylyl cyclase activity in coral tissues makes them a useful tool for identifying drugs that affect cAMP production. A previous screen for drugs that specifically targeted sAC identified a single inhibitor, KH7, from a collection of 15,312 lipophilic compounds (Hess et al., 2005). KH7 was

chosen as the best sAC inhibitor for its potency, high affinity, and specificity. No stimulators were identified from this screen. In comparison, this study identified three potential stimulators of cAMP production from a library of just 151 compounds derived from natural products. One of these compounds, laurencione monoacetate, was capable of stimulating purified rat sAC_t activity. Though experiments attempting to characterize its effect in live cells were inconclusive, it is clear that natural products are a valuable resource for discovering compounds with unique structures and pharmacological effects. The complexity of cAMP signaling pathways and the potential for cross-talk effects in live cells demonstrate the difficulties associated with characterizing compounds as novel pharmacological tools.

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