Schild analysis of the interaction between parthenolide and cocaine on their effects on planarian motility suggests an orthosteric relationship.

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One of the best-characterized planarian behaviors induced by various compounds is the change in locomotor velocity. Previous work from our laboratory showed that the sesquiterpene lactone parthenolide and the local anesthetic cocaine, reduce planarian motility. Parthenolide reverses the cocaine-induced motility decrease and vice versa. However, the exact mechanism of the cocaine/parthenolide antagonism for this specific planarian behavior is still unknown. Here we report the results of a Schild analysis to determine whether the parthenolide/cocaine relationship is orthosteric or allosteric. Our results suggest an orthosteric relationship between these two compounds in the planarian *Girardia tigrina*. The simplest interpretation of our data is a shared binding site for cocaine and parthenolide. Still, we cannot rule out the possibility of distinct yet overlapping binding sites with the data available.

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INTRODUCTION

Freshwater planarians have a distinguished history in regenerative and developmental biology research. This is an effort that has been ongoing for at least two centuries and is still a source of profound insights in these areas, particularly since the availability of molecular biology techniques, genomic analyses, and bioinformatics tools (Birkholz et al., 2018; Baguñà, 2018; Karami et al., 2015; Pagán 2014, 2017; Pellettieri, 2018; Robb et al., 2015). More recently, planarians have emerged as a powerful model to study regenerative processes, specifically in nervous tissue (Reviewed in Cebrià 2007, Gentile et al., 201; Owlarn and Bartscherer, 2016; Ross et al., 2017).

Planarians are also an emerging pharmacological model system for three main reasons: (1) the varied behavioral responses that these organisms exhibit upon exposure to exogenous substances, (2) the similarities of their nervous system to the nervous system of vertebrates in terms of cellular biology and morphology, and (3) the presence of vertebrate-like neurotransmitter systems in these organisms (Buttarelli et al., 2000; Ribeiro et al., 2005; Sarnat and Netsky, 1985; Pagán 2017). An especially active area of pharmacological research using planarians is to study the effect of abused drugs *in vivo*, including drugs such as cocaine, amphetamines, ethanol, and nicotine, among others (Bach et al., 2016; Byrne, 2018; Kusayama and Watanabe, 2000; Lowe et al., 2015; Pagán, 2017; Pagán et al., 2008, 2009, 2013, 2015; Raffa and Desai, 2005; Raffa and Valdez, 2001; Rawls et al., 2011; Rowlands and Pagán, 2008; Tallarida et al., 2014a,b).

The naturally-occurring sesquiterpene lactone parthenolide (Figure 1) is mainly isolated from the feverfew plant (*Tanacetum parthenium*). Feverfew is used in alternative medicine to treat migraines with some success, although the mechanism of action for this effect remains to be fully elucidated (Saranitzky et al., 2009). Parthenolide and other sesquiterpenoid

lactones are currently in clinical trials for their antitumoral properties, and are also being investigated for their anti-inflammatory effects (Chadwick et al., 2013; Ghantous et al., 2010, 2013; Mathema et al., 2012). However, parthenolide and related compounds are known to cause contact dermatitis (Nanda and Wasan, 2016), and studies on some other sesquiterpene lactones suggest genotoxic effects induced by these compunds (Amorim et al., 2013).

Our research group reported that parthenolide and related compounds antagonized the behavioral effects of cocaine exposure in planarians (Pagán et al., 2008; Rowlands and Pagán, 2008). Subsequently, we determined the structural correlates of parthenolide-like molecules for their cocaine-antagonist activity in planarians (Baker et al., 2011), and determined that parthenolide seems to be a specific antagonist of cocaine-induced seizure-like activity in these worms, as opposed to seizures induced by other abused substances such as nicotine or amphetamine (Pagán et al., 2012). Interestingly, parthenolide is able to antagonize certain cocaine-induced electrophysiological effects in rats (Schwarz et al., 2011), which validates the usefulness and vertebrate relevance of the planarian model.

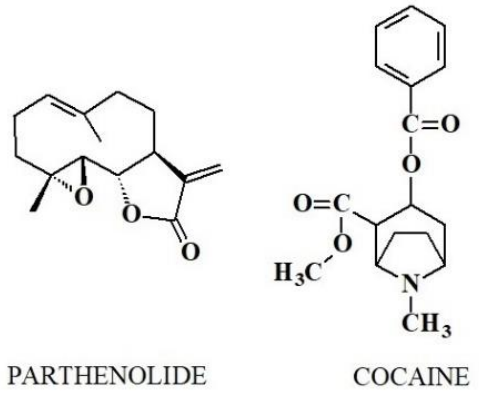

Figure 1. Compounds used in this work.

Although the molecular targets of cocaine in mammals are well-understood, very little is known about the mechanism of action and molecular nature of the cocaine target (s) in planarians. The work reported here provides evidence for an orthosteric relationship between cocaine and parthenolide for their ability to reduce motile behavior in the planarian *Girardia tigrina*.

MATERIALS AND METHODS

Brown planarians (*Girardia tigrina*) were purchased from Ward's (Rochester, NY). Parthenolide was purchased from Tocris (Minneapolis, MN), and cocaine from Sigma-Aldrich (St. Louis, MO). General laboratory materials, chemicals, and supplies were purchased from Fisher scientific (Suwanee, GA). All graphs and statistical analyses were done with the Prism software package (GraphPad Inc., San Diego, CA).

Upon receipt, the flatworms were transferred to artificial pond water (APW: 6 mM NaCl, 0.1 mM $NaHCO_3$, 0.6 mM $CaCl_2$), and were allowed to acclimate to laboratory conditions for at least 24 hours before using them for experiments. The worms were used within three weeks of arrival. The APW was changed at least once every day except during weekends and immediately before any experiments. All experiments were performed at room temperature in APW containing 0.1 % dimethylsulfoxide (DMSO) as a solubility-enhancing agent; this concentration of DMSO does not display any apparent behavioral or toxic effects in planarians (Pagán et al., 2006).

We used a modification of a published protocol to measure planarian motility (Raffa et al., 2001, as modified in Pagán et al., 2006; 2008). Briefly, using a small paintbrush or plastic pipette, a planarian (1.0-1.5 cm long) was placed in a transparent, APW-rinsed, 6 cm polystyrene Petri dish, the dish was placed over a 1 cm^2 grid, and five milliliters of APW/0.1 % DMSO (control) or 5 mL of the experimental solutions were added to the dish. The motility of the worm was measured by counting the squares crossed or re-crossed per minute for 5 minutes. Each worm was used only once. The data was then plotted as cumulative crosses *vs.* time and fit to a linear equation to obtain its slope. In experiments where the worms were exposed to various concentrations of the experimental compounds, the slopes obtained from the linear regression were normalized to control slopes (APW/0.1 % DMSO) and graphed as the fraction of control *vs*. the experimental compound concentration (Pagán et al., 2008). To analyze these concentration-response curves, we fit the data to an empirical Hill equation (Equation 1, Goutelle et al., 2008; Ortiz, 2013; Pagán et al., 2006) in the form:

$$F = IC_{50}^{n} / (IC_{50}^{n} + [Compound]^{n})$$
Equation 1

Where F is the fraction of control, [Compound] is the experimental compound concentration in μM, IC_{50} is the compound concentration that decreased planarian motility by 50 %, and n is the Hill coefficient.

We performed two sets of experiments to observe the effect of parthenolide or cocaine on the IC_{50} of the other to decrease planarian motility: A series of concentration-response curves of cocaine in the absence or the presence of single concentrations of parthenolide, and a series of concentration-response curves of parthenolide in the absence or the presence of single concentrations of cocaine. In these experiments, the compound used in the concentration-response curve was defined as "Compound A", and the compound added at a single concentration was defined as "Compound B". The chosen concentration of compound B did not induce motility decrease in planarians (Pagán et al., 2008). The shift in the IC_{50} for compound A, as induced by the single concentration of compound B, was subjected to Schild analysis by fitting the data to a linear equation (Equation 2) in the form:

$$\log((A'/A)-1) = m(-\log[Compound\ B]) + b$$
Equation 2

Where A and A' are the IC_{50} values obtained from a concentration-response curve of the first compound (e.g., cocaine) in the absence and in the presence of the second compound (e.g., parthenolide), respectively. The slope of this fit is called the *Schild slope*. A Schild slope not statistically different than unity is suggestive of an orthosteric relationship between the two tested compounds (Tallarida, 2007; Wyllie and Chen, 2007).

RESULTS AND DISCUSSION

Figures 2 and 3 show the concentration-response curves for planarian motility decrease induced by parthenolide in the absence and the presence of cocaine, and by cocaine in the absence and the presence of parthenolide, respectively. Tables 1 and 2 show the IC_{50} and Hill coefficient values and the Schild analysis data. For both sets of curves, the apparent IC_{50} of the first compound (A) increased in the presence of a single concentration compound B (Tables 1 and 2). Our data suggest a mutually-exclusive binding relationship between parthenolide and cocaine for their motility-decrease properties in planarians, consistent with previous studies from our laboratory (Pagán et al., 2008). However, the exact nature of this relationship is unknown. The simplest interpretation of this data would be that parthenolide and cocaine display an orthosteric interaction, (a single type of binding site shared by both compounds, or separate, yet overlapping binding sites for each compound).

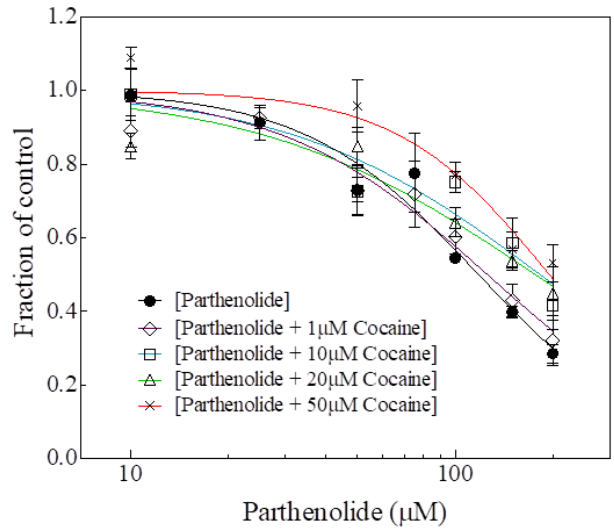

Figure 2. Concentration-response curves of motility decrease by parthenolide in the absence and in the presence of a single cocaine concentration, as indicated. Each data point represents the average of the responses of 8 worms. The lines were generated by fitting que data to Equation 1. The error bars represent the standard error of the mean. The Hill and Schild parameters are listed in Table 1.

Alternatively, the parthenolide/cocaine interaction could be allosteric, where each compound would have its own (yet related to the other's), binding site. We performed a Schild analysis (Arunlakshana and Schild, 1959) of the IC_{50} shift data to distinguish between these two possibilities, as outlined in the previous section. Briefly, if the value of the Schild slope is statistically equal to unity, the data would suggest an orthosteric relationship between parthenolide and cocaine. On the other hand, if the slope is significantly different from unity, the data would suggest an allosteric relationship between the two compounds (Colquhoun 2007; Hall and Langmead 2010; Raffa et al., 2006).

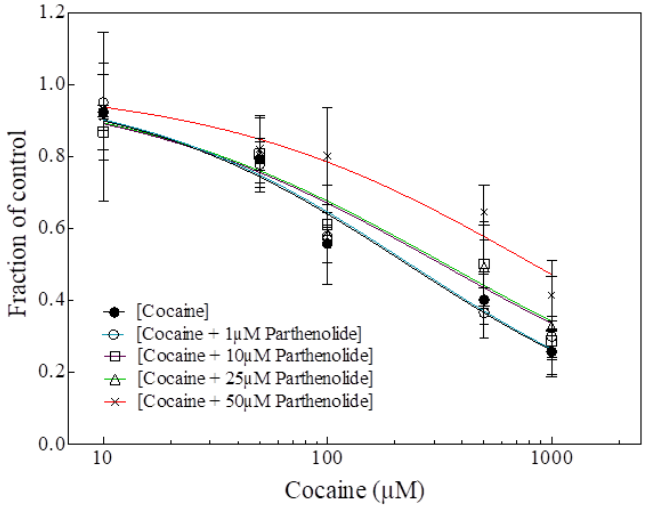

Figure 3. Concentration-response curves of motility decrease by cocaine in the absence and in the presence of a single parthenolide concentration, as indicated. Each data point represents the average of the responses of 8 worms. The lines were generated by fitting que data to Equation 1. The error bars represent the standard error of the mean. The Hill and Schild parameters are listed in Table 2.

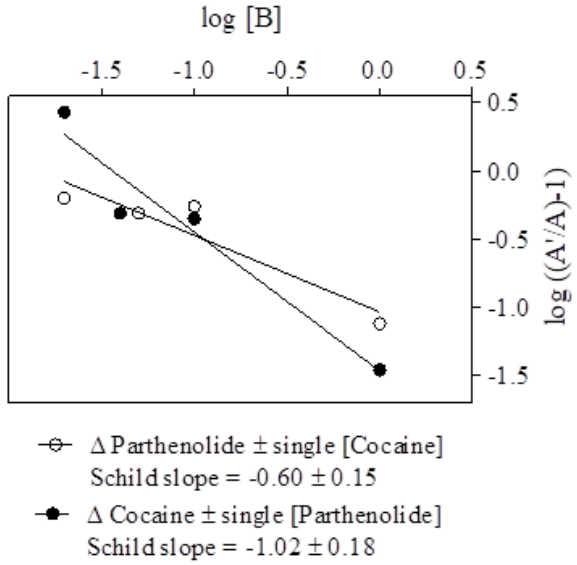

Figure 4. Schild plots of parthenolide and cocaine, as indicated (see text).

Figure 4 shows the Schild plots corresponding to the data from Figures 2 and 3, as summarized in Tables 1 and 2. For the parthenolide ± cocaine data, the Schild slope value is -0.60 ± 0.15, and for the cocaine ± parthenolide data, the Schild slope value is -1.02 ± 0.18. The linear fit parameters from each line were not significantly different from each other via an F-test ($F = 3.83$ and $p = 0.122$). At first glance, this statistical analysis seems to indicate an orthosteric relationship between the two experimental compounds. However, we wanted to confirm whether the Schild slopes for each compound were statistically different from -1. To determine this, we performed a one-sample t-test to assess whether the Schild parameter was different from a theoretical value of -1. The Schild slope for the parthenolide ± cocaine data (-0.60) was not statistically different from -1 ($p = 0.071$), and neither was the Schild slope for the cocaine ± parthenolide data (-1.02, $p = 0.919$). This being said, we need more experiments to verify if this data is robust enough to warrant our conclusions.

The best-understood molecular targets of cocaine in vertebrates are voltage-gated sodium channels (Tsuchiya, 2017), and the monoamine transporters (Immadisetty and Madura, 2013).

Planarians possess genes analogous to these proteins, based on genomic data available in various databases (Castillo-Lara and Abril, 2018; Nishimura et al., 2012; Robb et al., 2015), and the likeliest possibility would be that they will function analogously in planaria as they do in vertebrates. The Hill parameters for cocaine (Table 2), are significantly lower than 1, suggesting that cocaine acts on multiple types of binding sites in our experimental organism. This is consistent with the aforementioned types of targets of cocaine in vertebrate nervous systems. On the other hand, the Hill parameters for parthenolide (Table 1), are not significantly different from 1, suggesting a single class of sites. It is important to point out that the Schild and Hill parameters were developed to be used on isolated receptors or tissue, not explicitly for behavioral assays. Nonetheless, this could provide valuable information regarding the parthenolide and cocaine binding sites in planarians. We will explore the nature of these putative sites further by performing pharmacological dissections of these and alternate targets in future work

Cocaine induces other types of behaviors in planarians in addition to motility decrease. Some of these behaviors are expressed under acute exposure conditions, such as seizure-like hyperkinesia (Tallarida et al., 2014a,b), light/dark environmental preference (which is oftentimes interpreted as anxiety-

like responses; Nayak et al., 2016), and behavioral and cross sensitization (Rawls et al., 2010). Also, chronic exposure to cocaine induces conditioned place preference behaviors (Hutchinson et al., 2015; Tallarida et al., 2014a,b) and even withdrawal-like behaviors (Raffa and Desai, 2005; Raffa and Valdez, 2001). Of these cocaine-induced behaviors, we have demonstrated that parthenolide and related analogs antagonize cocaine-induced motility decrease and seizure-like hyperkinesia (Pagán et al., 2008, 2012) as well as withdrawal-like behaviors (Rowlands and Pagán, 2008). We have recently completed a study examining the effects of parthenolide and a related sesquiterpene lactone on planarian light/dark preference (Kakoturu et al., in preparation for submission). Still, mechanistic interpretations of the multiple cocaine effects in planarians and its antagonism by parthenolide are lacking. Also, the nature of their molecular target (s) is still unknown.

The present work is the first report of a mechanistic study of parthenolide/cocaine interactions, suggestive of an orthosteric relationship. The further study of such interactions will shed light on the mechanism of action of cocaine and perhaps other abused drugs. This information will surely contribute to a better understanding of how they cause their harmful effects in vertebrates, and will likely point to potential novel approaches to prevent such undesirable effects *in vivo*.

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Table 1. Hill and Schild parameters for motility decrease induced by parthenolide in the absence and in the presence of various cocaine concentrations (From Figure 2).

	Cocaine (no Parthenolide)	Cocaine (+ 1 μM Parthenolide)	Cocaine (+ 10 μM Parthenolide)	Cocaine (+ 25 μM Parthenolide)	Cocaine (+ 50 μM Parthenolide)
IC_{50} μM* ± SEM**	228 ± 26	236 ± 28	325 ± 51	340 ± 84	835 ± 277
Hill Coefficient ± SEM	0.7 ± 0.1	0.7 ± 0.1	0.6 ± 0.1	0.6 ± 0.1	0.6 ± 0.1
log ((A'/A)-1)***	---	-1.46	-0.371	-0.309	0.425
B (Parthenolide, μM)	---	1	10	25	50
-log B	---	0	-1	-1.4	-1.7

***From Equation 1 fit of the concentration-response curves (Figure 2).**
****Standard error of the mean.**
*****See text.**

Table 2. Hill and Schild parameters for motility decrease induced by cocaine in the absence and in the presence of various parthenolide concentrations (From Figure 3).

	Parthenolide (no Cocaine)	Parthenolide (+ 1 μM Cocaine)	Parthenolide (+ 10 μM Cocaine)	Parthenolide (+ 20 μM Cocaine)	Parthenolide (+ 50 μM Cocaine)
IC_{50} μM* ± SEM**	118 ± 8	126 ± 11	183 ± 29	176 ± 24	195 ± 18
Hill Coefficient ± SEM	1.6 ± 0.2	1.4 ± 0.2	1.1 ± 0.3	1.0 ± 0.2	1.9 ± 0.4
log ((A'/A)-1)***	---	-1.17	-0.259	-0.308	-0.185
B (Cocaine, μM)	---	1	10	20	50
-log B	---	0	-1	-1.3	-1.7

***From Equation 1 fit of the concentration-response curves (Figure 3).**
****Standard error of the mean.**
*****See text.**

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