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Reversal of cocaine-induced planarian behavior by parthenolide and related sesquiterpene lactones

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Abstract

Here we report the prevention and reversal of cocaine-induced behaviors in planarian worms by parthenolide and two related cyclic sesquiterpene lactones (SL), costunolide and santonin. Using established protocols, we studied two cocaine-induced behavioral effects in planaria; the induction of motility decrease and the induction of C-like hyperkinesia. Cocaine, parthenolide, costunolide, santonin, and a lactone-less cyclic sesquiterpene, β-eudesmol, decreased planarian motility in a concentration-dependent manner. Only cocaine induced C-like hyperkinesia. At concentrations that did not show any motility decrease, partenolide, costunolide and santonin, but not β-eudesmol, significantly reduced the cocaine-induced motility decrease and C-like hyperkinesia, in a concentration-dependent manner. Furthermore, partenolide, costunolide and santonin were able to rescue planaria from C-like hyperkinesia, after the worms were exposed to cocaine. Conversely, cocaine at a concentration that did not show any measurable effects (10 μM), was able to alleviate the SL-, but not the β-eudesmol-induced motility decrease. Liquid Chromatography/Mass Spectrometry experiments demonstrated that cocaine does not interact directly with any of the cyclic sesquiterpenoids, which suggests specific biochemical targets for these compounds in planarians. Our data suggests a common binding site for cocaine and the sesquiterpene lactones in planarians.

Keywords: Planaria; Cocaine; Parthenolide; Costunolide; Santonin; β-Eudesmol

1. Introduction

Cocaine addiction and toxicity have been intensively studied (Kreek et al., 2005); however, there are no established biological treatments for cocaine effects *in vivo* (Sofuoglu and Kosten, 2006). One strategy is to search for compounds capable of displacing cocaine from its molecular targets without interfering with normal physiology (Meltzer et al., 2002). Other efforts explore the role of vaccines (Martell et al., 2005) and antibodies (Carrera et al., 2005) targeted to the cocaine molecule. Enzymes such as cholinesterases (Pan et al., 2005) hydrolyze cocaine, producing an inactive metabolite, ecgonine methyl ester, which has been found to protect mice against cocaine toxicity (Hoffman et al., 2004).

Planarians are popular model organisms in developmental biology and regeneration research (Cebrià, 2007; Newmark and Sánchez Alvarado, 2002). Planarians also show promise in neuropharmacology research. They have a primitive nervous system, which shares many structural similarities with vertebrate nervous systems, including bipolar and multipolar neurons, dendritic spines (Cebrià, 2007; Sarnat and Netsky, 1985), and every major neurotransmitter found in vertebrates (Ribeiro et al., 2005; Sarnat and Netsky, 2002; Villar and Schaeffer, 1993). Planarians are being rediscovered as a useful animal model to study abused drugs, as these organisms display behavioral responses to psychoactive substances. For example, they show a decrease in motility after administration of the antipsychotic drug chlorpromazine (Stokely and Grossi, 1963) and display behaviors resembling tolerance and dependence in response to morphine (Needleman, 1967). Additionally, planarians exhibit behaviors resembling

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withdrawal symptoms upon exposure to cocaine (Raffa and Desai, 2005; Raffa and Valdez, 2001) and they show morphological changes in their brain and nervous system directly associated with chronic cocaine exposure (Margotta et al., 1997). In humans, one of the best understood effects of cocaine is the inhibition of neurotransmitter transporters, particularly the dopamine transporter (Torres et al., 2003). Interestingly, cocaine seems to interact with the dopaminergic system in planaria (Carolei et al., 1975; Palladini et al., 1996; Raffa et al., 2001). Planarians, therefore, provide a useful model to study cocaine effects and the effects of other abused drugs in biological systems.

Parthenolide and costunolide (Fig. 1) are germacranolides of the sesquiterpene lactone class of compounds (Fairbrothers et al., 1975). The germacranolides display herbicidal, fungicidal and bactericidal activity (Fischer et al., 1998; Macías et al., 1999; Wedge et al., 2000). These compounds also inhibit serotonin release from platelets (Marles et al., 1992) and show antitumoral and anti-inflammatory properties (Bocca et al., 2004; Kwok et al., 2001; Miglietta et al., 2004). Many sesquiterpene lactones are also inhibitors of the NF-kappaB transcription factor (Nam, 2006). Parthenolide is usually isolated from the feverfew plant (*Tanacetum parthenium*), which is used as an anti-migraine agent, but is also found in other plant species (Fraga, 2002). Parthenolide is also an inhibitor of serotonin type II receptors (Weber et al., 1997). Santonin is a compound structurally related to parthenolide and costunolide (Fig. 1), mainly isolated from the wormseed plant; santonin has been used and an antihelmintic agent (Birladeanu, 2003). β-Eudesmol (Fig. 1) is a cyclic sesquiterpene which lacks the lactone moiety. β-Eudesmol inhibits muscle-type nicotinic acetylcholine receptors (Kimura et al., 1991), has antiangiogenic activity (Tsuneki et al., 2005) and interestingly, is an antidote against organophosphate cholinesterase toxicity (Chiou et al., 1995).

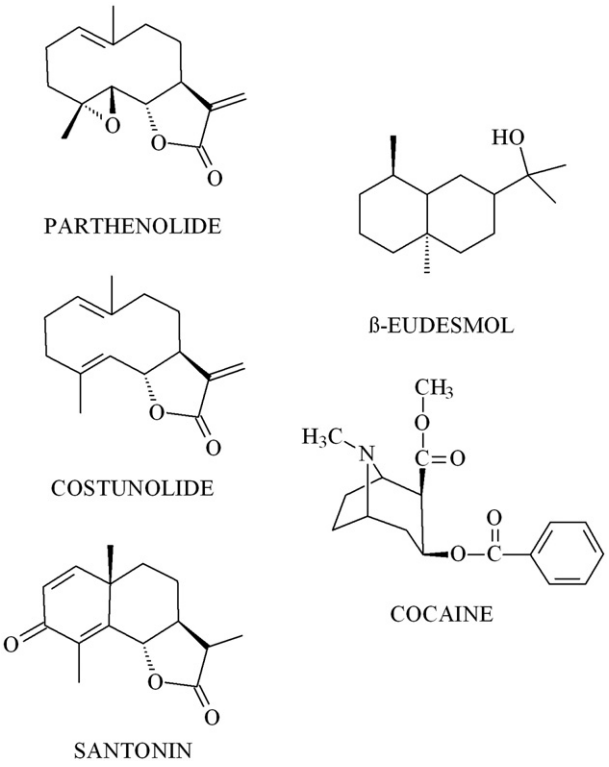

Fig. 1. Compounds used in this work.

In previous studies, parthenolide at a concentration of up to 1 mM did not inhibit acetylcholine-induced currents in the BC3H1 cell line, which express a muscle-type nicotinic acetylcholine receptor, yet was able to reverse the inhibition of such currents by cocaine (González, Pagán and Hess, unpublished observations). Moreover, in HEK-293 cells stably transfected with the human dopamine transporter (a kind gift from Dr. Jonathan Javitch, Columbia University), parthenolide and santonin were able to displace specifically bound [^{3}H]-cocaine, without significantly affecting dopamine uptake (Pagán, 2005). On the other hand, experiments using the aforementioned HEK-293, designed to reverse the functional cocaine inhibition of the dopamine transporter by parthenolide or santonin, proved inconclusive (Pagán, 2005). Since parthenolide and cocaine seem to interact in two different systems, we decided to explore the effects of cocaine, parthenolide and structurally-related compounds in planaria, using modifications of established behavioral protocols.

2. Methods

2.1. Animals and chemicals

Black planarian worms (*Dugesia dorotocephala*) were purchased from Ward's (Rochester, NY). General laboratory materials and supplies were from Fisher Scientific (Suwanee, GA) or Sigma-Aldrich (St. Louis, MO). The experimental compounds used are shown in Fig. 1. Parthenolide and costunolide were purchased from Tocris (Ellisville, MO). (−) Cocaine hydrochloride, β-eudesmol and santonin were purchased from Sigma-Aldrich (St. Louis, MO).

2.2. General procedures

Planarians were transferred to artificial pond water (APW, NaCl, 6 mM; $NaHCO_3$, 0.1 mM; $CaCl_2$, 0.6 mM; pH 6.9) upon receipt and left to acclimate to the laboratory conditions for at least 24 h before being used. The worms (1–1.5 cm long) were used within 2 weeks of arrival, and the APW was changed daily. The planarians were not fed at any time. All the experiments described in this work were in APW at room temperature and included 0.1% dimethylsulfoxide (DMSO) as a solubility-aiding agent. At this concentration, DMSO does not have any apparent behavioral or toxic effects in planaria (Pagán et al., 2006).

2.3. Motility experiments

To measure planarian motility, we used a modification of a published behavioral protocol (Raffa et al., 2001, as modified in Pagán et al., 2006). This is a simple, yet useful procedure that can be used to study the effects of experimental compounds on planarian locomotor behavior. Using a small paintbrush, a worm was gently transferred to an APW-rinsed 6 cm polystyrene dish and set on a grid (1 cm^2 squares, Fig. 2A), followed by adding 5 mL of APW including 0.1% DMSO with or without the

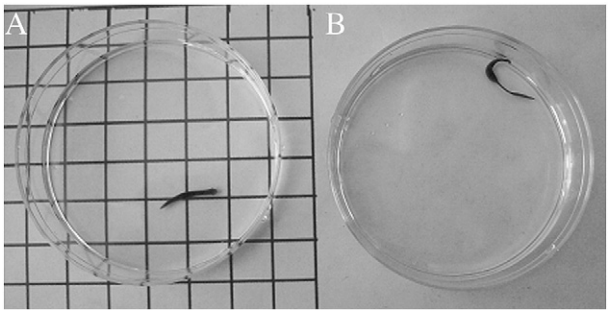

Fig. 2. A. Motility assay experimental setup (see text). B. Representative C-like position in response to cocaine exposure (see text).

experimental compounds. Initially, we chose a cocaine concentration (100 μM) similar to a concentration (80 μM) which was previously reported to decrease planarian locomotor velocity approximately by half upon pre-exposure (Raffa et al., 2001) and tested the effect of the incubation time of cocaine on its ability to decrease planarian locomotor velocity. Planarian motility was measured by counting each time the worm crossed a square, minute by minute, over a period of 8 min. Each worm was used only once. This data was graphed as cumulative crosses *vs.* time, and fit to a linear equation. In experiments where the worms were exposed to increasing concentrations of the experimental compounds, the slopes obtained by the linear equation fit were normalized to control slopes and plotted as the fraction of control *vs.* the experimental compound concentration. To analyze our concentration–effect curves, we fit the data to an empirical Hill-type equation (Eq. (1)) in the form:

$$F = \mathrm{IC}_{50}^{n} / \left(\mathrm{IC}_{50}^{n} + [\mathrm{compound}]^{n}\right) \quad (1)$$

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. This is the best initial treatment of the data, since we have no information about the possible mechanism of action of cocaine and the other compounds in planaria. The Hill coefficient can indicate cooperativity between two or more binding sites. Generally, Hill coefficients significantly higher or lower than unity indicate positive or negative cooperativity, respectively, provided that the putative binding sites differ on their affinity for a specific ligand. Two different binding sites with similar affinities for the ligand would be indistinguishable with this equation. Even in binding studies, however, the Hill equation provides little or no information about the specific mechanism (Weiss, 1997). This is primarily a behavioral work; in a whole organism there will be multiple binding sites which will interact to induce specific behaviors. In this context, n is meaningless and therefore it is not examined here.

2.4. Cocaine-induced C-like hyperkinesias

Planaria exhibit C-like hyperkinesias in response to exposure to cocaine, amphetamines and dopamine (Fig. 2B, Palladini et al., 1996). We examined the induction of this behavior by increasing concentrations of cocaine in the absence and in the presence of the other experimental compounds. A worm was transferred to a 6 cm dish as described in Section 2.3, in the absence (control) and in the presence of the experimental compounds. The C-like positions were counted for a period of 30 min in the absence or in the presence of 10, 25 or 50 μM of the cyclic sesquiterpenoids, and graphed as a function of cocaine concentration. The data points were fit to a rectangular hyperbola-like equation (Eq. (2)), in the form:

$$C = (P^{*}[\mathrm{Cocaine}]) / (K + [\mathrm{Cocaine}]) \quad (2)$$

where C indicates the number of C-like positions in 30 min, P is the Y-axis plateau, [Cocaine] is the cocaine concentration in μM and K is a constant.

To assess the ability of the cyclic sesquiterpenoids to rescue planaria from cocaine pre-exposure, a series of experiments were done in which the C-like positions were counted in worms exposed to 500 μM cocaine for a period of 15 min, followed by the administration of 25 μM of the other experimental compounds for 15 additional minutes.

2.5. Liquid Chromatography/Mass Spectrometry (LC/MS)

We used LC/MS to rule out the possibility of a direct interaction of cocaine with the other experimental compounds. For that purpose, we tested solutions of cocaine in the absence and in the presence of equimolar concentration of each of the tested sesquiterpenes, using a Waters model HP 1100 (Waters Corp., Milford, MA) with a built-in autosampler. The separations were accomplished on a Luna C18(2) column (150×2 mm, 5 μm; Phenomenex, Torrance, CA) . The sample chamber in the autosampler was maintained at 4 °C, while the column was at 30 °C. The mobile phase consisted of 10 mM ammonium acetate in water

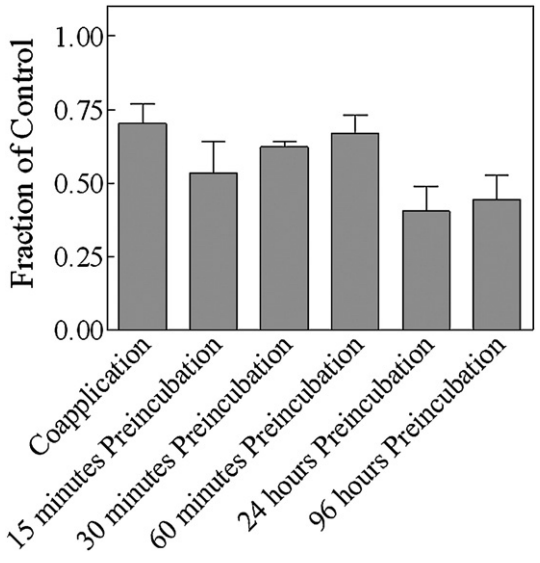

Fig. 3. The cocaine-induced motility decrease in planarians is independent of the time of exposure. The decrease in motility induced by 100 μM cocaine was examined as a function of exposure time, as indicated. There was no significant difference between any of the exposure times ($p>0.05$ by the Kruskal–Wallis test — nonparametric ANOVA). Each bar represents the average of three to nine separate measurements. Error bars represent the standard error of the mean.

(A) and 90 mM methanol in water (B) and was delivered at 0.5 mL/min. The linear gradient conditions were as listed in Table 3. The mass spectrometer used was a Micromass Quattro Ultima triple quadrupole mass spectrometer (Waters Corp.), equipped with an electrospray interface and operated in the positive mode ionization mode. Settings for the mass spectrometer are listed in Table 4. LC/MS/MS analysis was performed by Full scan MS mode, the semi-quantitative concentration of cocaine and other drugs were obtained. Micromass MassLynx software (version 4.0, Waters Corp.) was used for analysis of LC/MS data.

2.6. Statistical analysis

All graphs and statistical procedures were done using the Prism 5/InStat software package (GraphPad Software, San Diego, CA).

3. Results

3.1. Effect of the experimental compounds on planarian motility

Fig. 3 shows a series of experiments measuring planarian motility in the presence of 100 μM cocaine. Motility measurements were taken immediately after exposure (coapplication) or after preincubating for up to 96 h, as indicated. In all cases, the planarians slowed down to about 50% of the control. We chose a preincubation time of 15 min in all subsequent motility experiments.

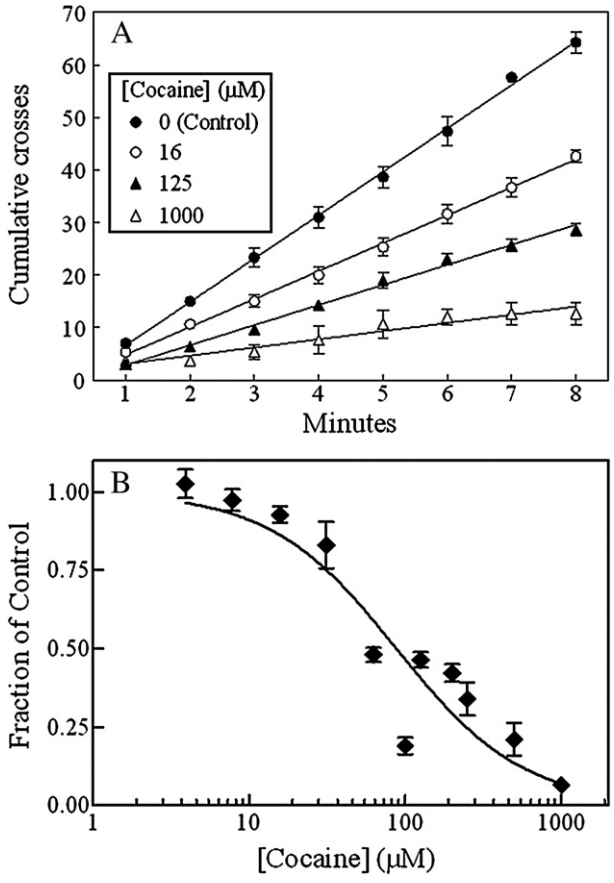

Fig. 4. A. Representative experiment showing the effect of cocaine in planarian motility. The data points were fit to a linear equation to generate the plots. B. Concentration–response curve of the cocaine-induced decrease on planarian motility. The line was generated by fitting the data to Eq. (1). The fit parameters are shown in Table 1. In both graphs, each data point includes the average of three to five worms and the error bars represent the standard error of the mean.

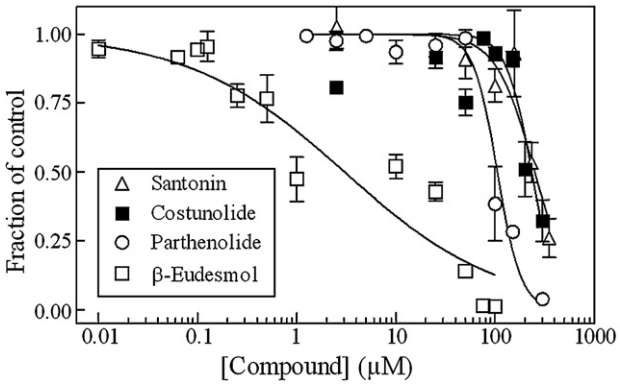

Fig. 5. Concentration–response curves of the effect of the cyclic sesquiterpenoids on planarian motility, as indicated. The lines were generated by fitting the data to Eq. (1). The fit parameters are shown in Table 1. Each data point includes the average of 8 to 11 worms. Error bars represent the standard error of the mean.

Fig. 4A shows a series of plots of cumulative crosses (see Section 2.3) *vs.* time, in the absence (control) and in the presence of representative cocaine concentrations. In control experiments, the worms typically crossed 1-cm wide gridlines at a rate of 7 to 8 gridlines per minute (Fig. 4A, closed circles). This agrees closely with previous reports (Raffa and Valdez, 2001; Raffa et al., 2006) where planarians were observed to cross 0.5-cm wide gridlines at a rate of 15 to 16 gridlines per minute under control conditions. This figure also shows a clear concentration-dependence on the cocaine's ability to slow down the worms. By normalizing the experimental slopes with the control slopes we constructed a concentration–response curve of cocaine-induced motility decrease as a function of cocaine concentration (Fig. 4B). We followed this procedure for all further motility experiments.

All the tested compounds decreased planarian motility in a concentration-dependent manner (Figs. 4B, and 5). Their IC_{50} values are shown in Table 1. The concentration-dependent cocaine-induced motility decrease in planaria is consistent with published reports (Palladini et al., 1996). They found that

Table 1
The IC_{50} values for planarian motility decrease by the tested compounds

Compound tested	IC_{50} (μM±s.e.m.[a])	*p*-value[b]
Parthenolide	105±5	–
Costunolide	233±13	<0.01
Santonin	250±31	<0.001
β-Eudesmol	3.0±0.7	<0.01
Cocaine	88±11	>0.05

These values were obtained by fitting the data from Figs. 4B and 5 to Eq. (1) (see text).

[a] Standard error of the mean.

[b] Comparison of the IC_{50} value for parthenolide with the IC_{50} value for all the other experimental compounds (Tukey–Kramer Multiple Comparisons Test; Motulsky, 1995, 1999).

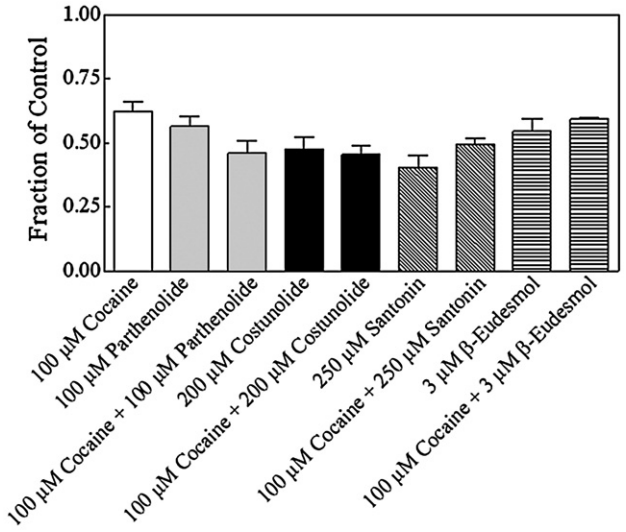

Fig. 6. The motility decrease induced by cocaine or the cyclic sesquiterpenoids is not synergistic. The planaria were exposed to cocaine or the cyclic sesquiterpenoids separately or in combination, as indicated. Even though the variation among bars seems to be greater than expected by chance ($p<0.05$ by nonparametric ANOVA), none of the bars were significantly different from each other ($p>0.05$, Dunn's Multiple Comparisons Test). Error bars represent the standard error of the mean.

planaria exposed to cocaine for 2 h became motionless at a concentration range of 59–589 μM, while worms exposed to 1.5 μM cocaine displayed normal motility.

3.2. The motility decrease induced by cocaine and the cyclic sesquiterpenoids is not synergistic

Based on the IC_{50} values obtained for each of the experimental compounds (Table 1), we observed the motility decrease induced by cocaine and the cyclic sesquiterpenes by themselves or in combination (Fig. 6). There was no significant difference in any of the treatments, meaning that the combination of a cyclic sesquiterpene and cocaine, both at concentrations that individually decrease planarian motility by 50% did not exacerbate the response of the other.

3.3. Parthenolide, costunolide and santonin, but not β-eudesmol, decreased the apparent potency of cocaine to decrease planarian motility

Fig. 7 shows a series of parallel experiments of cocaine effects on planarian motility in the absence and in the presence of 50 μM of parthenolide, costunolide, santonin or 0.1 μM β-eudesmol. At these concentrations, none of the sesquiterpenes significantly decreased planarian motility (Fig. 5). The

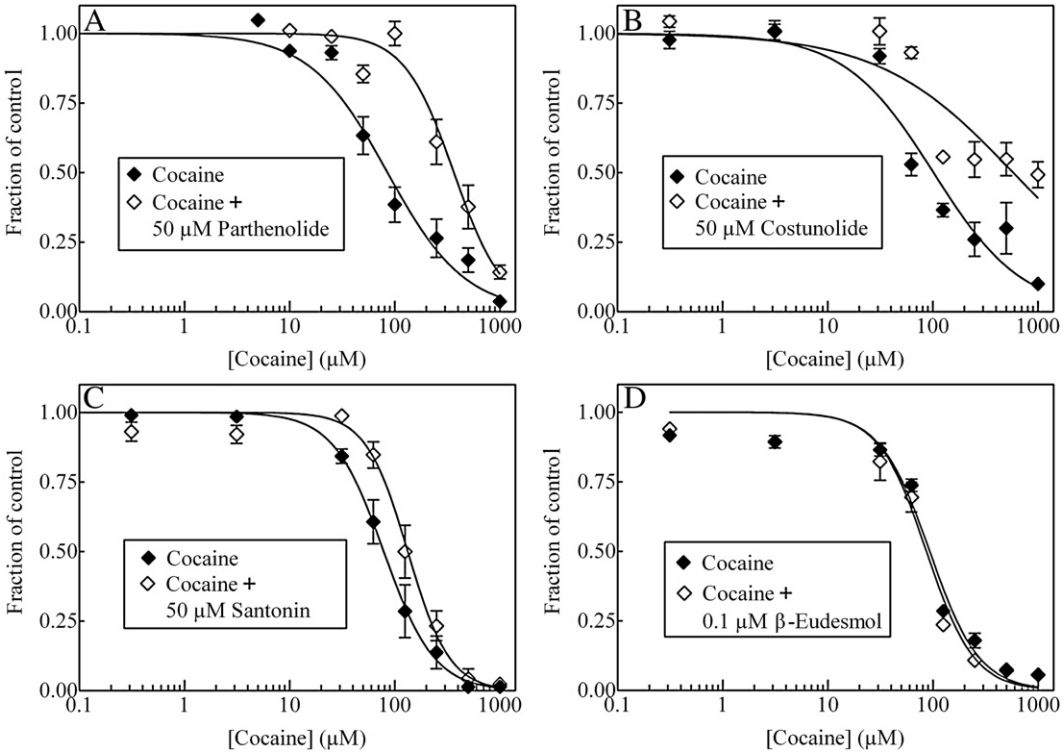

Fig. 7. The sesquiterpenes parthenolide, costunolide and santonin, but not β-eudesmol, diminished the apparent potency of cocaine to decrease planarian motility. The figures show parallel curves of cocaine inhibition of planarian motility in the absence (closed symbols) and in the presence (open symbols) of the indicated concentrations of the tested compounds. All three sesquiterpene lactones significantly increased the IC_{50} of cocaine inhibition of planarian motility (Table 2, *F*-test; Motulsky, 1995, 1999). A. Parthenolide ($p<0.0001$), B. Costunolide ($p<0.0001$), C. Santonin ($p<0.0001$). β-Eudesmol (D), was not able to alleviate the cocaine effect ($p=0.947$). None of the cocaine-alone curves were significantly different from each other ($p>0.05$). The lines were generated by fitting the data to Eq. (1); the fit parameters are shown in Table 2. Each data point includes the average of 5–10 worms. Error bars represent the standard error of the mean.

Table 2
The IC_{50} values for cocaine decrease in planarian motility in the absence and in the presence of the tested cyclic sesquiterpenes (see text)

Compounds tested	IC_{50} (μM±s.e.m.[a])	F[b]	p-value[b]
Cocaine	85±9		–
Cocaine+50 μM parthenolide	365±32	(2, 108)≡55.9	<0.0001
Cocaine	102±13		–
Cocaine+50 μM costunolide	580±132	(2, 60)≡29.6	<0.0001
Cocaine	79±5		–
Cocaine+50 μM santonin	125±10	(2, 50)≡18.2	<0.0001
Cocaine	93±6		–
Cocaine+0.1 μM β-eudesmol	81±6	(2, 44)≡1.16	0.323

These values were obtained by fitting the data from Fig. 7 to Eq. (1) (see text).
[a]Standard error of the mean.
[b]Comparison of the IC_{50} value for cocaine with the IC_{50} value for cocaine in the presence of the indicated concentration of the second compound (F-test; Motulsky, 1995, 1999). None of the cocaine-alone curves were significantly different from each other (F: 6, 133≡1.95; $p>0.05$).

presence of any of the sesquiterpene lactones significantly increased the IC_{50} value for cocaine (Fig. 7A, B, C; Table 2). Parthenolide, costunolide and santonin induced a 4.3, 5.7 and 1.6-fold increase in the cocaine IC_{50} respectively (Table 2). In contrast, the presence of 0.1 μM β-eudesmol did not shift the concentration–response cocaine curve (Fig. 7D, Table 2).

The SL alleviation of cocaine-induced decrease in planarian motility was concentration-dependent. Fig. 8 shows the motility decrease induced by 100 μM cocaine in the absence and in the presence of increasing cyclic sesquiterpene concentrations; 100 μM cocaine decreased planarian motility to 25–45% of control. In each case, SL concentrations below 10 μM did not significantly alleviate the motility decrease induced by 100 μM cocaine. At concentrations of 25 and 50 μM, parthenolide completely alleviated cocaine effects while costunolide and santonin alleviated cocaine effects to 60–80% of control (Fig. 8). Similar experiments of cocaine in the absence and in the presence of β-eudesmol, did not prevent the motility decrease induced by 100 μM cocaine (Fig. 8).

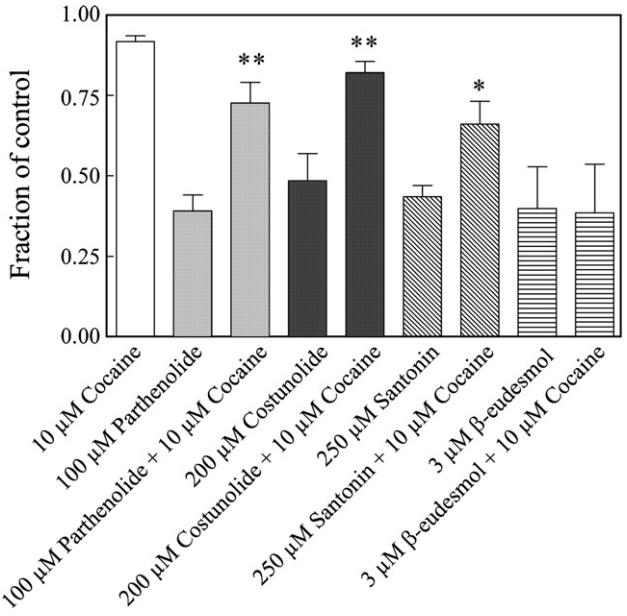

Fig. 9. Cocaine at a concentration of 10 μM alleviates the motility decrease induced by parthenolide, costunolide and santonin, but not β-eudesmol. We tested these data by split-plot repeated measures ANOVA, with "Treatment" (parthenolide, costunolide, santonin or β-eudesmol) as a "between-subjects factor" and with *vs*. without added cocaine as the "repeated measures factor". The effect of cocaine is highly significant ($p<0.001$), and the interaction is highly significant ($p=0.003$) as well, while the effect of any of the cyclic sesquiterpenoids by themselves is not different from each other ($p=0.261$). Based on these results, we followed the analysis by two-tailed, paired t-tests for each of the drugs, with or without cocaine. The effect of parthenolide, costunolide or santonin in the absence or in the presence of 10 μM cocaine is significantly different ("**", $p<0.01$; "*", $p<0.05$). On the other hand, cocaine did not affect the motility decrease caused by β-eudesmol ($p>0.05$). Each bar represents the average of 8–10 worms. The error bars represent the standard error of the mean.

3.4. Cocaine, at a concentration of 10 μM, prevented the sesquiterpene lactone-, but not the β-eudesmol-induced motility decrease in planaria

Fig. 9 shows a set of experiments of SL-induced motility decrease in the presence and in the absence of 10 μM cocaine. At

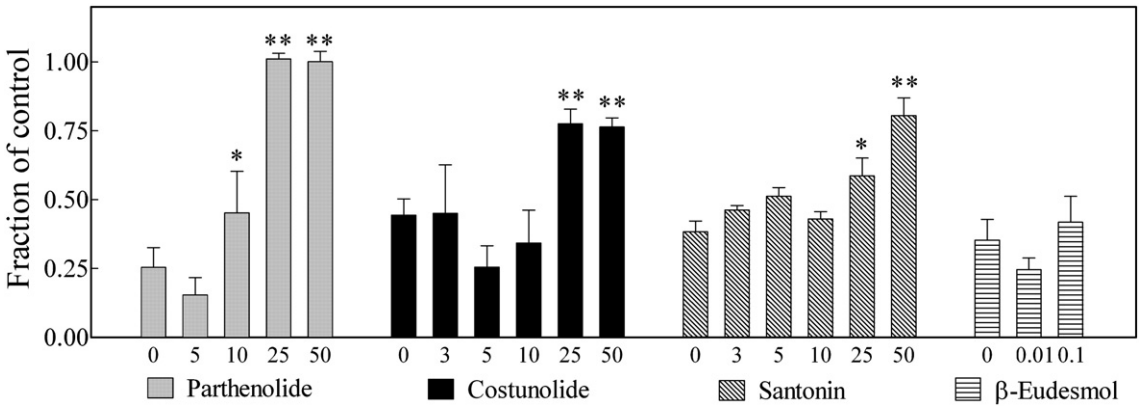

Fig. 8. The sesquiterpene lactones, but not β-eudesmol, alleviated the motility decrease in planarians induced by 100 μM cocaine in a concentration-dependent manner. The bars labeled "0" show the motility decrease induced by 100 μM cocaine. These four "0" bars were not significantly different from each other ($p>0.05$; Dunn's Multiple Comparisons Test; Motulsky, 1995, 1999). The other bars represent the effect of 100 μM cocaine in the presence of the indicated μM concentrations or parthenolide, costunolide, santonin or β-eudesmol, as indicated ("**", $p<0.01$; "*", $p<0.05$; Dunn's Multiple Comparisons Test; Motulsky, 1995, 1999). Each bar represents the average of 7–10 worms. The error bars represent the standard error of the mean.

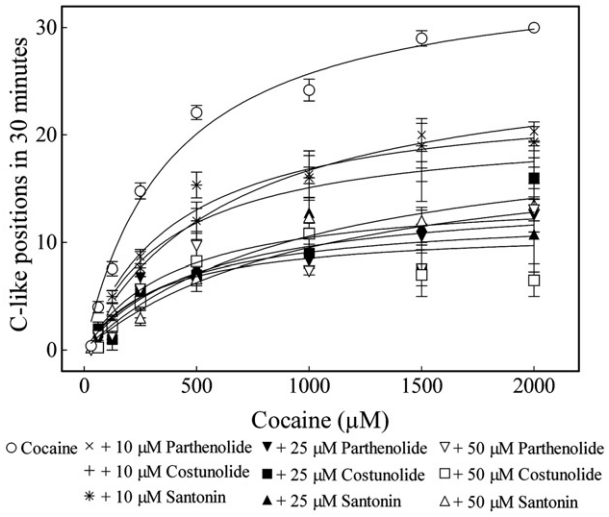

Fig. 10. Cocaine induces C-like hyperkinesia in planarians in a concentration-dependent manner. This effect is prevented by parthenolide, costunolide and santonin, as indicated. None of the sesquiterpene lactones nor β-eudesmol, induced C-like hyperkinesia up to a concentration of 100 μM (data not shown). Additionally, β-eudesmol did not alleviate the cocaine-induced C-like hyperkinesia (data not shown). The lines were generated by fitting the data points to Eq. (2). Each data point represents the average of 5 to 10 worms. Error bars represent the standard error of the mean.

this concentration, cocaine did not significantly decrease planarian motility. The sesquiterpenes were tested at concentrations close to their IC_{50} value; 100, 200, 250 and 3 μM for parthenolide, costunolide, santonin and β-eudesmol respectively. At these concentrations, each experimental compound decreased planarian locomotion to about 50% of the control. Cocaine was able to reverse the motility decrease in planaria elicited by parthenolide, costunolide and santonin. On the other hand, cocaine was unable to reverse the motility decrease induced by 3 μM β-eudesmol.

3.5. Cocaine-induced C-like hyperkinesia experiments

Fig. 10 shows a series of experiments of the induction of C-like hyperkinesias as a function of cocaine concentration in the absence and in the presence of 10, 25 or 50 μM parthenolide, costunolide or santonin, as indicated. None of the sesquiterpenes induced C-like hyperkinesias up to a concentration of 100 μM (data not shown). On the other hand, the presence of any SL significantly decreased cocaine's ability to induce C-like movements; 10 μM of any of the SL (but not β-eudesmol, data not shown) reduced these movements roughly by half, while 25 or 50 μM reduced the C-like positions by more than 75%. Fig. 11 shows that parthenolide, costunolide and santonin, but not β-eudesmol, were able to rescue planarians from cocaine-induced C-like hyperkinesia when the worms were pre-exposed to cocaine.

3.6. Liquid Chromatography/Mass Spectrometry (LC/MS)

The HPLC linear gradient conditions and the mass spectrometer settings are shown in Tables 3 and 4 respectively. The full scan mass spectrometer conditions are shown in Table 5. Fig. 12 and Table 6 show that the cocaine signal is unaffected by the presence of equimolar concentrations of any of the cyclic sesquiterpenoids.

4. Discussion

Here we report the effect of cocaine and four cyclic sesquiterpenoids, parthenolide, costunolide, santonin and β-eudesmol (Fig. 1), on selected aspects of planarian motility. To our knowledge, this is the first report of the interaction of cocaine and cyclic sesquiterpenoids *in vivo*. Our results indicate that cocaine and the tested sesquiterpenes elicit a concentration-dependent decrease in locomotor behavior (Fig. 4B, and 5), while

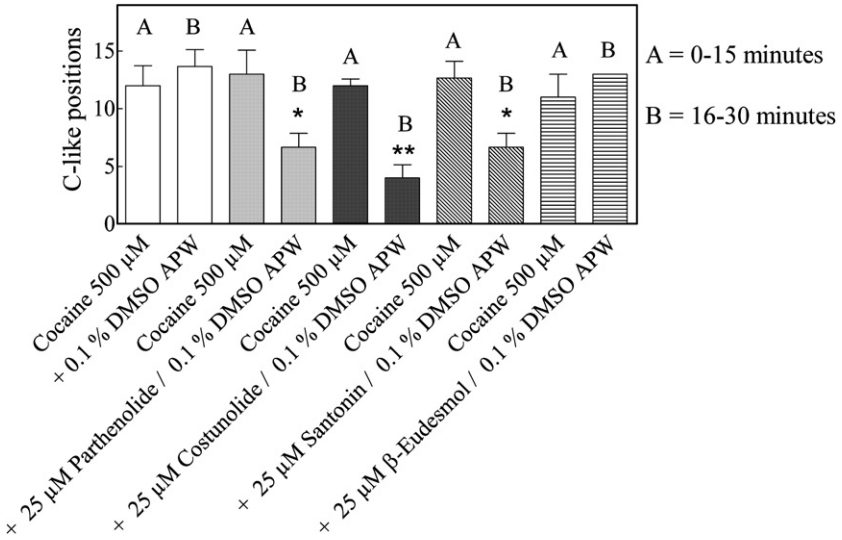

Fig. 11. The sesquiterpene lactones, but not β-eudesmol reverse the C-like hyperkinesia induced by pre-exposure of 500 μM cocaine. All the bars labeled "A" represent the number of C-like positions counted in the presence of 500 μM cocaine in the first 15 min, followed by "B", the administration of the vehicle (0.1% DMSO, white bars) or 25 μM of the other experimental compounds in 0.1% DMSO, as indicated, counting the C-like positions from minute 16 to 30. Each bar represents the average of three trials ("**", $p<0.01$; "*", $p<0.05$; two-tailed paired *t*-test; Motulsky, 1995, 1999). Error bars represent the standard error of the mean.

Table 3
HPLC linear gradient conditions

Time (min)	10 mM ammonium acetate (%)	90 mM methanol (%)
0	90	10
4.0	50	50
8.0	35	65
10.0	5.0	95
11.0	5.0	95
11.1	90.0	10
15.0	90.0	10

only cocaine induced C-like hyperkinesia (Fig. 10). Furthermore, we report that parthenolide, costunolide and santonin, reduced the apparent potency of cocaine to induce its behavioral effects. In contrast, a lactone-less cyclic sesquiterpenoid, β-eudesmol (Fig. 1), decreased planarian motility and did not alleviate any of the described cocaine-induced behavioral effects.

Planarians display two main types of locomotor movements, gliding and crawling (Pearl, 1903). Gliding is modulated by ciliary action, thought to be independent from the nervous system (Jenkins, 1967). Crawling is mainly modulated by muscular contraction when the worms are stimulated (Jenkins, 1967). A recent work (Nishimura et al., 2007) reported that dopaminergic innervation regulates locomotion and other motion-related behaviors in the planarian *Dugesia japonica*. In addition, planarians are affected by amphetamines, which are compounds that, like cocaine, affect the dopamine transporter. Planarians show preference to environments containing amphetamines (Kusayama and Watanabe, 2000) and their motility is increased in the presence of such compounds (Raffa and Martley, 2005). In the planarian, *D. japonica*, metamphetamine induced hyperkinesia in amputated heads, but not in the trunk and tail sections (Nishimura et al., 2007). The amphetamine-induced hyperkinesia returns to the trunk sections upon regeneration of the head (Nishimura et al., 2007). This strongly suggests a site of action for amphetamine within the planarian central nervous system. It would be interesting to explore the relationship between amphetamines and the sesquiterpene lactones in planarians. In vertebrates, the dopaminergic system is affected by amphetamines and cocaine (Torres et al., 2003).

The fact that cocaine and the tested cyclic sesquiterpenoids were capable of inducing behavioral effects in planaria in a concentration-dependent manner suggests that there are binding sites for these compounds in this organism. Moreover, the fact that parthenolide, costunolide and santonin were able to prevent the cocaine-induced motility decrease, again in a concentration-dependent manner (Figs. 7 and 8), suggests that the putative binding sites for cocaine and the sesquiterpene lactones are related. Of the tested sesquiterpene lactones, costunolide seems to be the most potent compound capable of preventing cocaine effects in this experimental setup. Costunolide not only right-shifted the apparent cocaine IC_{50} approximately six-fold (Table 2), but also the cocaine inhibition of planarian motility in the presence of costunolide never went below 50% at the highest cocaine concentration (1000 μM), which is approximately ten times the IC_{50} of cocaine alone (Table 1). However, 50 μM costunolide did not completely prevent the motility decrease induced by 100 μM cocaine, in contrast to parthenolide, which completely prevented such motility decrease (Fig. 8). Parthenolide displayed intermediate potency; it was able to increase the apparent cocaine IC_{50} approximately four-fold. Santonin was the least potent of the tested sesquiterpene lactones, increasing the apparent cocaine IC_{50} approximately two-fold (Fig. 7, Table 2).

Table 4
Mass spectrometer settings

ESI spray	3.5 kV
Cone	45 V
Mass resolution of scanning mass analyzer	0.7 Da±0.2 Da width at half height
Mass resolution of non-scanning mass analyzer for LC/MS experiments	1–2 Da width at half height
Desolvation gas flow	900–1100 L/h
Cone gas flow	50–70 L/h
Source block temp.	120 °C
Desolvation gas temp.	300 °C

Table 5
Full scan MS conditions for cocaine and other drugs

Compound	Precursor ion (*m/z*, nominal mass)
Cocaine	289.13
Parthenolide	264.17
Costunolide	248.19
Santonin	262.16
β-Eudesmol	224.21

The experiments with β-eudesmol indicate that a lactone ring associated to the ten-carbon cycle is not necessary to elicit the observed motility decrease. Moreover, the motility decrease curve of β-eudesmol is clearly biphasic (Fig. 5), which suggests a more complex mechanism of action. On the other hand, β-eudesmol was not able to alleviate the motility decrease induced by 100 μM cocaine (Fig. 7D) nor was it able to increase the IC_{50} value for cocaine in concentration–response curves (Fig. 8). This indicates that a lactone moiety may be necessary to antagonize the cocaine effect, information which might provide novel direction for the design of anti-cocaine agent candidates. More examples of cyclic sesquiterpenes need to be studied. The evident relationship between the cyclic sesquiterpenes and cocaine binding sites is further supported by the experiments where the motility decrease induced by parthenolide, costunolide and santonin was reversed by 10 μM cocaine (Fig. 9). Again, the exception was β-eudesmol. Since β-eudesmol does not seem to interact with cocaine, it seems that it decreased planarian motility through a different mechanism or biochemical target.

Further evidence linking the putative cocaine and SL binding sites in planarians is shown in the C-like experiments, where is shown that the SLs, but not β-eudesmol, are also able to antagonize the cocaine effects in a concentration-dependent manner (Fig. 10). Additionally, we demonstrate that the SLs, but not β-eudesmol are able to rescue planaria from C-like hyperkinesia when the worms were pre-exposed to 500 μM cocaine, followed by the administration of the vehicle (0.1% DMSO), or 25 μM of the cyclic sesquiterpenoids in 0.1% DMSO (Fig. 11). These data shows that the SLs act as true alleviators of cocaine effects in our experimental organism.

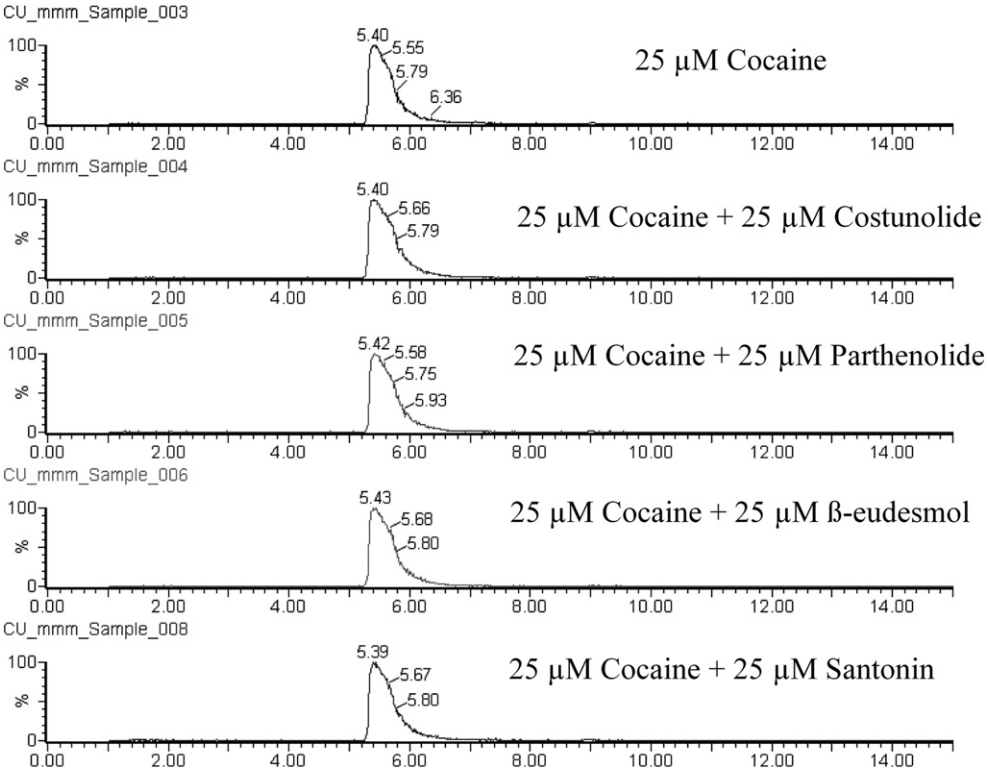

Fig. 12. Liquid Chromatography/Mass Spectrometry experiments. Measurements were taken with the indicated solutions. The experimental parameters are found in Tables 3–6.

Cocaine and the cyclic sesquiterpenoids do not seem to interact with each other directly. A careful look at Fig. 7 reveals that at almost any point, the sesquiterpenoid lactones are able to overcome cocaine effects. This is more pronounced for costunolide (Fig. 7B), where we show that 50 µM is capable of preventing about 50% of the effect induced by 1000 µM cocaine. These molecules are very similar with respect to their molecular sizes (Table 5). This point is reinforced by the C-like experiments (Fig. 10) in which we show that a SL concentration as low as 10 µM is capable of preventing 30–40% of the C-like positions induced by 2000 µM cocaine. Additionally, Fig. 11 shows that 25 µM of any of the SLs is able to rescue about 50% of the normal movement in worms pre-exposed to 500 µM cocaine. A direct interaction between the SLs and cocaine is extremely unlikely to say the least. Nevertheless, to further demonstrate that these two classes of compounds do not interact directly with each other, we did Liquid Chromatography/Mass Spectrometry experiments, which are shown in Fig. 12 and Tables 3–6. Fig. 12 and Table 6 clearly shows that the cocaine signal is unchanged in the presence of equimolar concentrations of any of the other experimental compounds. Taken together, our data indicates that cocaine and the studied cyclic sesquiterpenoids do not interact directly with each other; rather, they seem to induce their behavioral effects in planarians through specific binding sites. Moreover, the binding sites for cocaine and the sesquiterpene lactones seem to be related. Experiments aimed at the biochemical characterization of the radiolabeled cocaine binding sites in planaria are currently underway in our laboratory.

Table 6
Full scan MS peak area of different ions

Solution	Peak area
APW	ND[a]
0.1% DMSO APW	ND[a]
25 µM cocaine/0.1% DMSO APW	1.34E+8
25 µM cocaine+25 µM costunolide/0.1% DMSO APW	1.30E+8
25 µM cocaine+25 µM parthenolide/0.1% DMSO APW	1.31E+8
25 µM cocaine+25 µM β-eudesmol/0.1% DMSO APW	1.33E+8
25 µM cocaine+25 µM santonin/0.1% DMSO APW	1.30E+8

[a] Not determined.

The specific nature and pharmacology of these putative cocaine binding sites in planaria are yet unknown. However, all the available information points at close neurochemical similarities between the planarian and vertebrate nervous systems, suggesting a high level of phylogenetic conservation. For example, planaria displays vertebrate-relevant pharmacology with the neurotransmitters dopamine (Kitamura et al., 1998; Nishimura et al., 2007; Venturini et al., 1989), acetylcholine (Buttarelli et al., 2000) and with cannabinoids and opioids (Buttarelli et al., 2002) among others. Using microarray technology, a series of genes expressed in planarian brains was found to be homologous to genes coding for vertebrate nervous system proteins, such as glutamate and acetylcholine receptors (Cebrià et al., 2002; Nakazawa et al., 2003). Voltage-gated calcium channels (Cobbett and Day, 2003), caffeine-sensitive ryanodine receptors (Day et al., 2000) and serotonin receptors (Saitoh et al., 1996) have also been identified in planarians. A planarian genome project, using the planarian *Schmidtea mediterranea*, is underway (Robb et al.,

2007). This database is posted at http://smedgd.neuro.utah.edu/. Using this website, we have found several protein candidates homologous with dopamine, serotonin and norepinephrine transporters, as well as with sodium channels. These proteins are the usual cocaine targets in vertebrates (Scholz, 2002; Torres et al., 2003). As stated above, cocaine and other abused drugs interact closely with dopaminergic pathways. Planarians, therefore, are useful research subjects to study the effects of cocaine in biological systems, alongside with the fruit fly *Drosophila melanogaster* and the nematode, *Caenorhabditis elegans*, invertebrates that have been used to study the abused drugs cocaine, ethanol and nicotine (Rothenfluh and Heberlein, 2002; Wolf and Heberlein, 2003).

In summary, our results indicate that the sesquiterpene lactones are capable of reversing behavioral effects induced by acute cocaine exposure in planarians. The apparent lack of interaction between cocaine and β-eudesmol, hints at the importance of the lactone moiety for this effect. Our data suggests a common binding site for cocaine and the sesquiterpene lactones in planarians. The study of the sesquiterpene lactones might provide novel directions for the design of anti-cocaine agent candidates.

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