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Short communication

The non-competitive blockade of $GABA_A$ receptors by an aqueous extract of water hemlock (*Cicuta douglasii*) tubers

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ABSTRACT

Water hemlocks (*Cicuta* spp.) are acutely toxic members of the Umbellierae family; the toxicity is due to the presence of C_{17}-polyacetylenes such as cicutoxin. There is only limited evidence of noncompetitive antagonism by C_{17}-polyacetylenes at $GABA_A$ receptors. In this work with WSS-1 cells, we documented the noncompetitive blockade of $GABA_A$ receptors by an aqueous extract of water hemlock (*Cicuta douglasii*) and modulated the actions of the extract with a pretreatment of 10 μM midazolam.

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Water hemlocks (*Cicuta* spp.) are acutely toxic members of the Umbellierae, or carrot family, that grow in wet habitats such as streambeds or marshlands, and have been considered one of the most toxic plants of North America for many years (Kingsbury, 1964). Water hemlocks contain a variety of toxic C_{17}-polyacetylenes of which the most significant is cicutoxin (Uwai et al., 2000). Aqueous extracts of *Cicuta maculata* green seeds or tubers containing C_{17}-polyacetylenes have been shown to be acutely toxic to mice (Panter et al., 2011). Cicutoxin has been shown to increase the duration of *Lymnaea stagnalis* giant neuron repolarization (Wittstock et al., 1997). Cicutoxin has a mouse LD_{50} value of 2.8 mg/kg when dosed intraperitoneally and an IC_{50} value of 0.5 μM for the displacement of 4-n-[^{3}H]propyl-4′-ethynylbicycloorthobenzoate ([^{3}H]EBOB) from a neuronal membrane preparation isolated from rat cerebral cortex (Uwai et al., 2000). [^{3}H]EBOB binds to the noncompetitive antagonist site of the $GABA_A$ receptor also known as the picrotoxin binding site. These observations provide evidence that cicutoxin and related C_{17}-polyacetylenes act as noncompetitive antagonists of the $GABA_A$ receptor by binding to the picrotoxin binding site within the chloride channel to block ion flow through the channel (Ratra et al., 2001; Chen et al., 2006; 2011; Olsen, 2006). The related C_{17}-polyacetylene oenanthotoxin from *Oenanthe crocata* (hemlock water dropwort) was first pharmacologically characterized by Grundy and Howarth (1956) by comparing its actions in mice and rabbits to those of picrotoxin. This observation has been used as evidence for cicutoxin action at GABA receptors.

Poisoning by water hemlock is characterized by violent convulsions and ultimately respiratory failure in livestock and humans. In humans, the onset of symptoms can occur in as little as 15 min or up to 10 h after consumption and persist for up to 96 h (Schep et al., 2009). Treatment of poisoning includes airway management and seizure control through the use of benzodiazepines and barbiturates (Schep et al., 2009). Although water hemlock is acknowledged to be one of the most acutely toxic plants in North America, relatively little research has been done to pharmacodynamically characterize the toxic principles of this plant. Moreover, Schep et al. (2009) has suggested that there is only limited evidence as to the noncompetitive actions of cicutoxin at GABA receptors in the central nervous system (CNS). In this work, we characterized the actions of an aqueous extract of water hemlock (*Cicuta douglasii*) at

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$GABA_A$ receptors expressed by WSS-1 cells and modulated the actions of the extract with midazolam, a benzodiazepine.

Tubers from *C. douglasii* were collected about 12 km southwest of Naf, ID (N41 58.244 W113 25.536). The tubers were rinsed and stored at −80 °C until use. An aqueous extract of the tuber was made as previously described (Panter et al., 2011) and then centrifuged at 3000 rpm for 15 min to pellet any remaining solids. An aliquot of the extract was prepared for GC–MS analysis as previously described (Panter et al., 2011) by extraction with ethylacetate and conversion to the trimethylsilyl ethers for detection of cicutoxin and cicutol-type compounds.

A membrane potential sensing dye assay was used to measure the change in cell membrane potential, not the absolute value of the membrane potential (Leishman and Waldron, 2006). For this assay, WSS1 [WS1] (ATCC® CRL2029™) cells were obtained from ATCC (Manassas, Virginia) and cultured as described on the ATCC product sheet (ATCC, 2013). WSS-1 cells express functional $GABA_A$ receptors (Wong et al., 1992). The cells were grown to near confluence and then transferred to black well, clear bottom 96-well assay plates (Corning Incorporated, Corning, NY) 12–18 h prior to the assay.

Molecular Devices blue dye (Sunnyvale, CA) was reconstituted as previously described (Green et al., 2010) and on the day of assay, a prealiquoted tube of blue dye was thawed to room temperature and a 96 well compound plate was prepared using an aqueous water hemlock extract at 1, 5, 10, 15 and 20% of a 100 μL final volume. The water was evaporated off of the extract at room temperature and then resuspended in 100 μL of the blue dye solution. The WSS-1 cells were allowed to come to room temperature, culture medium aspirated, and replaced with the blue dye,-hemlock extract solution from the above plate and equilibrated for 30 min before being placed on a Flexstation 3 plate reader (Molecular Devices, Sunnyvale, CA) and varying concentrations of γ-aminobutyric acid (GABA, Tocris Bioscience, Bristol, United Kingdom) or receptor modulators (Sigma, St. Louis, MO) in blue dye solution were added to the cells by the flexstation. Flexstation readings and data analysis were performed as previously described (Green et al., 2010) with the exception that the baseline reading was taken at 18 s into the experiment.

In the concentration-effect experiments, the cellular responses to GABA in the presence and absence of the water hemlock extract were normalized to the maximum dye response generated by 50 μM GABA. Fifty percent effective concentrations and maximal responses were determined using a sigmoidal dose-response

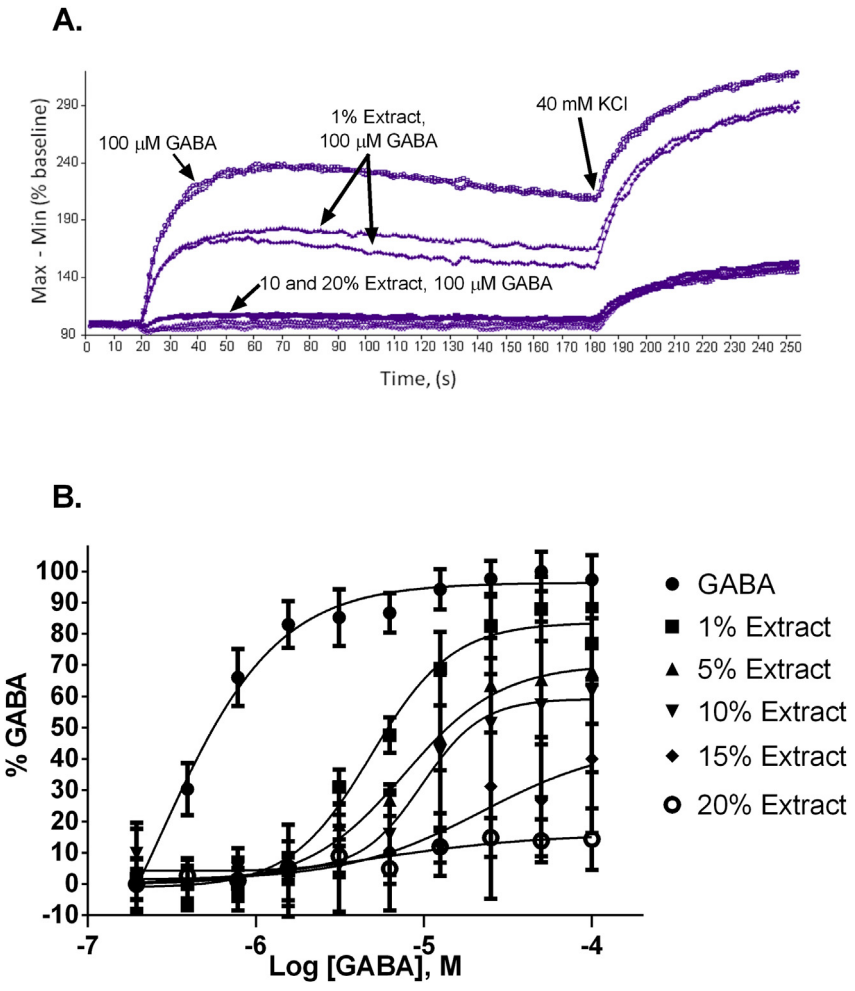

Fig. 1. The actions of an aqueous water hemlock extract on the GABA response of WSS1 cells. (A) The change in fluorescence of a membrane potential sensing blue dye over 255 s in response to 100 μM GABA in the presence or absence of 0, 1, 10 and 20% water hemlock extract (extract) from the same 96 well plate experiment. The solutions containing the test compounds were added at times indicated by arrows in duplicate wells. Forty mM KCl blue dye solution added at 180 s was used as a depolarizing calibrant to correct for interwell differences in dye loading and cell count. (B) The actions of 0, 1, 5, 10, 15 and 20% water hemlock extract on the membrane depolarization resulting from the addition of GABA in log10 M concentrations was measured and displayed as a percentage of the 50 μM GABA response. Each data point represents the mean ± S.E.M. of four to seven experiments of duplicate wells.

Table 1
EC_{50} values and maximum GABA responses in the absence (GABA Control) and presence of a water hemlock extract.

Percent extract	EC_{50} value (μM)	EC_{50} 95% confidence interval (μM)	Percent maximal response ± std. error
GABA control	0.3	0.03–2	96 ± 4
1%	5	3–7	84 ± 6
5%	8	3–17	70 ± 11
10%	10	3–28	59 ± 13
15%	22	2–292	45 ± 26
20%	6	0.1–212	16 ± 11

equation log (agonist) vs. response-variable slope (four parameters) with Prism version 6.03 (GraphPad Software, La Jolla California USA, www.graphpad.com). The benzodiazepine and barbiturate drug treatments, water hemlock extract and 100 μM GABA experiments were normalized to the 100 μM GABA response for each respective extract concentration. Multiple comparisons were made with a one-way ANOVA and a Tukey's multiple comparisons test with Prism. The limit for statistical significance was set at $P < 0.05$.

The chemical analysis of the extract detected the presence of cicutoxin and cicutol-type compounds (Supplemental Figure 1). Representative tracings of the membrane potential sensitive dye fluorescence from duplicate wells of the GABA control and wells pretreated with 1, 10 and 20% water hemlock extract (W.H.) are displayed in Fig. 1A. The concentration–effect relationship for GABA (n = 7) and the GABA concentration–effect relationships in cells pretreated with 1 (n = 5), 5 (n = 6), 10 (n = 5), 15 (n = 6), and 20 (n = 6) % W.H. are displayed in Fig. 1B. The 50 μM GABA response was used for the normalization of all membrane potential fluorescence responses in Fig. 1B. Note the depression of the maximum GABA responses in the W.H. pretreatment experiments that is characteristic of non-competitive antagonism. The EC_{50} values, EC_{50} value 95% confidence intervals, and percent maximal responses for GABA and the 1, 5, 10, 15 and 20% W.H. pretreatment concentration-effect experiments are presented in Table 1. The ability of benzodiazepines and barbiturates to reverse the W.H. induced depression of the WSS-1 cell response to 100 μM GABA was also examined (Fig. 2). Pretreatment with 5% W.H. significantly reduced the 100 μM GABA responses. When WSS-1 cells were pretreated with a combination of individual benzodiazepines or barbiturates in the presence of 1 or 5% W.H., only 10 μM midazolam significantly reversed the effects of the extract.

In the mammalian central nervous system, GABA and the $GABA_A$ receptor are the major inhibitory amino acid neurotransmitter and receptor respectively. These receptors are members of the Cys-loop family of ligand-gated anion channels and are comprised of five subunits arranged around a central pore (for review see Sigel and Steinmann, 2012). $GABA_A$ receptors serve to provide inhibitory tone to the CNS (Semyanov et al., 2004). Cicutoxin blockade of the $GABA_A$ receptors inhibits GABA central nervous system tone causing the seizures observed in poisoned individuals (Schep et al., 2009).

One of the aims of this work was to pharmacodynamically characterize an extract of water hemlock. When WSS-1 cells were pretreated with an aqueous extract of water hemlock tubers, the maximum WSS-1 cell GABA responses were depressed in a concentration-dependent manner which is characteristic of non-competitive antagonism (Fig. 1B) (Kenakin, 2009). Uwai et al. (2000) using radioligand binding experiments provided evidence that cicutoxin, like picrotoxin, binds to the noncompetitive antagonist site in the channel pore of the $GABA_A$ receptor to block ion flow. Results presented in this work with an aqueous extract of water hemlock tubers provide functional cell-based experimental evidence that an aqueous extract from water hemlock tubers which contains cicutoxin and cicutol-type compounds (Supplemental Figure 1) noncompetitively blocks $GABA_A$ receptors.

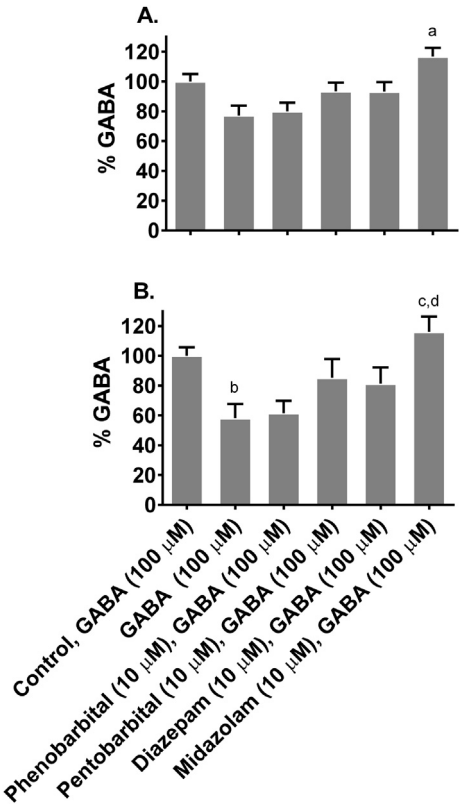

Fig. 2. The affects of the barbiturates and benzodiazepienes on inhibition of the 100 μM GABA response in the absence (control) or presence of either 1% panel (A) or 5% panel (B) water hemlock extract in WSS1 cells. Each bar represents the mean ± S.E.M. of eight experiments of duplicate wells with 1% water hemlock extract and seven experiments of duplicate wells with 5% water hemlock extract and all treatments responses were normalized to their respective control, GABA response. [a]P < 0.05 midazolam pretreatment vs. the 100 μM GABA response in the presence of 1% extract. [b]P < 0.05 control 100 μM GABA vs. the 100 μM GABA response in the presence of 5% extract. [c]P < 0.05 midazolam pretreatment vs. the 100 μM GABA response in the presence of 5% extract. [d]P < 0.05 midazolam pretreatment vs. the 10 μM phenobarbital, 100 μM GABA response in the presence of 5% extract.

In addition to characterizing the nature of the water hemlock extract antagonism, we also tested the ability of barbiturates and benzodiazepines to modulate the actions of the hemlock extract on the GABA response. In this work, midazolam, a benzodiazepine, significantly reversed the actions of the 1% and 5% aqueous extracts (Fig. 2AB). In rats, midazolam can delay the onset of picrotoxin induced seizures (Hasan et al., 2014). Both picrotoxin and cicutoxin displace [^{3}H]EBOB from the noncompetitive antagonist binding site on the $GABA_A$ receptor (Kume and Albin, 1994; Uwai et al., 2000), suggesting that the mechanism of midazolam receptor modulation may be similar for both toxins and that midazolam may have clinical utility in treating water hemlock intoxications. It would also be useful to determine if midazolam in combination with inhibitors

of GABA metabolism like vigabatrin could decrease the severity of seizures due to water hemlock poisoning in experimental animal models.

Conflict of interest

The authors declare that there are no conflicts of interest.

Ethical statement

The authors declare that this manuscript complies with the Elsevier Ethical Guidelines for Journal Publication.

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Appendix A. Supplementary figure

Supplementary figure related to this article can be found at http://dx.doi.org/10.1016/j.toxicon.2015.09.015.

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