Pergamon

Bioorganic & Medicinal Chemistry Letters, Vol. 6, No. 9, pp. 1025-1028, 1996

0960-894X/96 $15.00 + 0.00

S0960-894X(96)00166-7

INHIBITION OF PROTEIN PHOSPHATASE 2A BY CANTHARIDIN ANALOGUES.

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Abstract: The syntheses of several cantharidin analogues and their biochemical effects on protein phosphatase 2A are described. Comparison of the PP2A inhibition of these cantharidin analogues has shown that the 7-oxo moiety and the anhydride system contribute to the biochemical activity. The diacid forms of several anhydrides showed reduced inhibition of PP2A. Copyright © 1996 Elsevier Science Ltd

Cantharidin (exo,exo-2,3-dimethyl-7-oxobicyclo[2.2.1]heptane-2,3-dicarboxylic acid anhydride) **1** is a naturally occurring toxin present in over 1500 different species of the Chinese blister beetle (*Mylabris phalerata* or *M. cichorii*).[1,2] The toxin has also been isolated from the Spanish fly (*Cantharis vesicatoria*).[2] The dried body of the Chinese beetle was first used by the people of China as a traditional medicine over 2000 years ago.[1] The toxin was isolated in Europe during the 1800's from the Chinese blister beetle and used in the treatment of warts, as an aphrodisiac and an abortifacient.[2,3] By the early 1900's cantharidin was determined too toxic for use as an internal medicine.[3] It is now known that oral ingestion leads to severe irritation and ulceration of the gastrointestinal and urinary tract epithelial linings and that intraperitoneal injection causes severe congestion and edema of the liver with a LD_{50}=1mg/kg.[2b,4] Cantharidin has a long history of poisoning in both animals and humans. Livestock toxicosis due to the consumption of feed containing the blister beetles continues to present a problem for ranchers.[5] Several human poisonings have recently been reported as a result of ingestion of the toxin for its supposed aphrodisiac qualities.[6]

Cantharidin was found to bind, with high affinity, to a specific cantharidin-binding protein (CBP) isolated from mouse liver.[7] The CBP had high homology with PP2A (AC type) and showed significant phosphorylase a phosphatase activity which was inhibited by okadaic acid as well as cantharidin.[7] Okadaic acid also inhibited the specific [^{3}H]cantharidin binding to CBP, identifying the protein as a type of protein phosphatase.[7] Subsequently, the inhibitory activity of cantharidin was compared with okadaic acid on the activity of the purified catalytic subunits of PP1, PP2A and PP2B.[8] The results showed that like okadaic acid (IC_{50} 0.4nM for PP2A, C type), cantharidin inhibited PP2A at a lower concentration (IC_{50} 0.16μM) than PP1 (IC_{50} 1.7μM), and that PP2B is inhibited only at much higher concentrations (IC_{50} >1000μM). This established that cantharidin was a member of the okadaic acid class compounds.[8,9] The okadaic acid class is represented by six structural types: okadaic acid[10], calyculin A[11], microcystin-LA[12], nodularin[13], tautomycin[14] and cantharidin. Cantharidin is

particularly interesting as it is the smallest, least flexible compound within the class and there is limited structure activity data available.

We synthesized thirteen cantharidin analogues following standard methods.[15] The compounds were tested for their inhibitory activity against partially purified PP2A (heterotrimeric complex ABC type) isolated from mouse brain.[16] The heterotrimeric complex was chosen to allow correlation with data previously reported for the okadaic acid class compounds. (ref) The IC_{50} value of cantharidin against PP2A (ABC type) was determined to be 8μM (Figure 1). The thirteen cantharidin analogues were screened for the inhibition of PP2A at a concentration of 1mM. Three showed greater than 80% inhibition of PP2A, two showed approximately 50% inhibition and the remaining showed less than 40% inhibition (Table 1). The IC_{50}'s of the three active compounds **2**, **3** and **4** were determined to be 40μM, 70μM and 300μM, respectively (Figure 2).

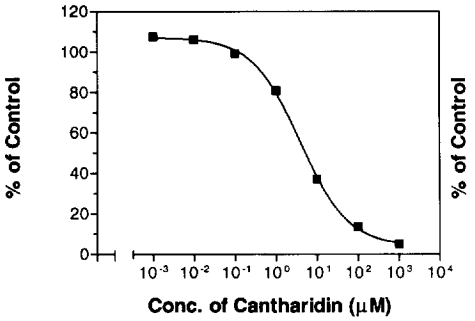


Figure 1: Inhibition of Protein Phosphatase Activity. The inhibitory effect of cantharidin on the activity of partially purified PP2A from mouse brain. The assay was conducted using [^{32}P]phosphorylase a as substrate. Incubation was conducted for 10 minutes at 30 °C. Protein phosphatase activity was determined by the liberation of [^{32}P]phosphate into the supernatant after precipitation of protein with trichloroacetic acid. Each point represents the mean of duplicate measurements.

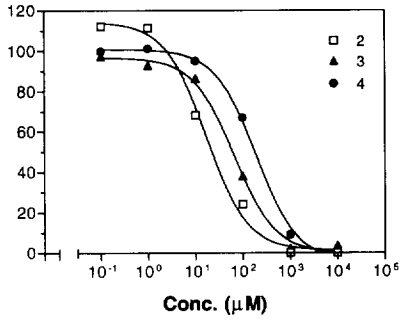


Figure 2: Inhibition of Protein Phosphatase Activity. The inhibitory effects of the cantharidin analogues 2, 3 and 4 on the activity of partially purified PP2A from mouse brain. The assay was conducted using [^{32}P]phosphorylase a as substrate. Incubation was conducted for 10 minutes at 30 °C. Protein phosphatase activity was determined by the liberation of [^{32}P]phosphate into the supernatant after precipitation of protein with trichloroacetic acid. Each point represents the mean of duplicate measurements.

Compounds **2**, **3** and **4** were the most potent of the thirteen cantharidin analogues. The three compounds contained a 7-oxo moiety and lacked the bridgehead methyls. Compounds **2** and **3** contained an anhydride system, like cantharidin. Ring opening of the anhydride of **2** to give **4** resulted in a 7.5-fold decrease in activity. Cantharidin was still the most potent compound. Comparison of these four compounds indicated that the presence of the bridgehead methyls was not essential for activity and that the presence of the double bond, although increasing the rigidity of the ring system, did not significantly affect activity. The compounds **5** and **6** both showed approximately 50% inhibition at a concentration of 1mM. Comparison of these compounds with the more active analogues indicated that the presence of a 7-oxo is essential for potent activity. The stereochemistry of these two compounds (**5** is endo,endo and **6** is exo,exo) had little effect on their activity. The inactive compounds either lacked the 7-oxo moiety or lacked the bridge altogether.

The inhibition of CBP activity by several cantharidin analogues has been recently reported.[7] The results are summarized in Table 2. All analogues contained the 7-oxo moiety and were dicarboxylic acids. The three most potent compounds, cantharidin, cantharidic acid and 2,3-trimethylene anhydride contained either bridgehead methyls at positions 2 and 3 or a bridging 2,3-trimethylene group. Removal of the methyl group at position 3, and of both bridgehead methyls of cantharidic acid resulted in a decrease in potency. Removal of the bridgehead

methyls and addition of a carboxylic acid at position 5 of cantharidic acid also resulted in a decrease in potency. Substitution at positions 1 and 4, or position 5 of endothall resulted in a significant decrease in binding to CBP.

Table 1: Inhibition of PP2A Activity by Cantharidin Analogues.

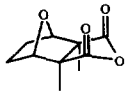
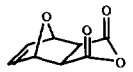
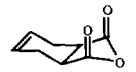
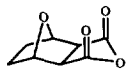
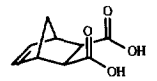
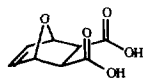
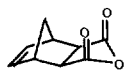
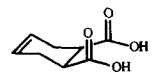
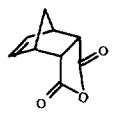
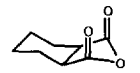
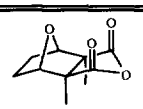
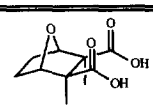
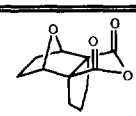
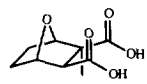
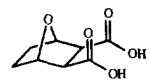
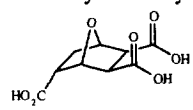
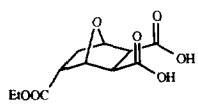
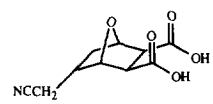
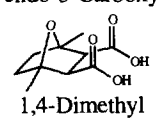
Compound	Inhibition	IC50	Compound	Inhibition	IC50
1 	95.0%	8μM	**8** 	30.8%	ND
2 	95.2%	40μM	**9** 	24.0%	ND
3 	88.3%	70μM	**10** 	17.2%	ND
4 	80.3%	300μM	**11** 	13.3%	ND
5 	48.1%	ND	**12** 	12.2%	ND
6 	48.8%	ND	**13** 	9.2%	ND
7 	38.7%	ND	**14** 	3.8%	ND

Table 2: CBP Phosphatase and [^{3}H]Cantharidin Binding Inhibition by Other Cantharidin Analogues.[7]

Phosphatase inhibition	IC50 for radioligand binding	Cantharidin (anhydride) and endothall (dicarboxylic acid) analogues		
92-95%	4-8nM	 Cantharidin	 Cantharidic acid	 2,3-Trimethylene anhydride
22-51%	10-50nM	 2-Methyl	 Endothall	 endo-5-Carboxy
0%	200 to >100000nM	 endo-5-Carboethoxy	 endo-5-Cyanomethyl	 1,4-Dimethyl

Our study showed that removal of the bridgehead methyls resulted in a decrease in potency. The inhibition of PP2A (ABC type) by cantharidin (**1**) was decreased 9-fold in the 'bisnor' compound **3.** This was similar to the 6-fold decrease observed for inhibition of CBP by cantharidic acid and endothall. Importantly, we have shown that the 7-oxo moiety is required for inhibition of PP2A. Comparing compounds **2** and **5**, there is a decrease in inhibition as a result of the conversion of the 7-oxo moiety to a 7-methylene. Removal of the bridge of **2** and **5**, with consequent stereochemical changes, to give **9** resulted in a further loss of activity. Similar

results were also observed for the two series of compounds (**3**, **7** and **13**) and (**4**, **10** and **12**). Cantharidin and cantharidic acid exhibited similar activity with respect to enzyme inhibition of CBP. Our results indicated, however, that ring opening of the anhydride system of **2** to give the diacid **4** resulted in a significant reduction in inhibition of PP2A. Similar results were observed for the compounds (**5** or **6** and **10**) and (**9** and **12**). In a subsequent experiment, we determined the IC_{50} values for cantharidin and cantharidic acid to be 4 and 32 μM respectively. The anhydride **2** (δ 6.49, 5.30, 3.27 ppm) in 50 mM Tris-HCl buffer (pH 7.0) / d_6-DMSO (1:1) showed complete conversion to the diacid **4** (δ 6.37, 5.03, 2.71 ppm) over 40 min indicating that the inhibitory values of the anhydrides may be underestimates.

Acknowledgements: We thank the Australian Research Council and the Monbusho International Scientific Research Program from the Ministry of Education, Science and Culture, Japan for support of this research. We acknowledge the award of an Australian Postgraduate Award to CT.

References and Notes

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16 Protein phosphatase 2A was partially purified and assayed in a modification of the method previously reported in Nishiwaki, S.; Fujiki, H.; Suganuma, M.; Nishiwaki-Matsushima, R.; Sugimura. T. *FEBS Lett.* **1991**, *279*, 115.
Protein phosphatase 2A was isolated from mouse brain by DEAE-cellulose column chromatography using 50mM Tris-HCl buffer (pH 7.4). Partially purified PP2A was eluted with buffer containing 0.2 M NaCl. Enzyme activity was measured in 50 mM Tris-HCl buffer (pH 7.0) containing 100 μM EDTA, 5 mM caffeine, 0.1% 2-mercaptoethanol, 0.6 mg/mL BSA and [^{32}P]phosphorylase a. Inhibition of protein phosphatase activity was determined by incubation of [^{32}P]phosphorylase a (5 μg), protein phosphatase 2A (11.8 μg) and various concentrations of cantharidin or cantharidin derivatives for 10 min at 30°C in 100 μL volume in duplicate. The reaction was terminated by addition of 100 μL of ice cold 50% trichloroacetic acid. After centrifugation an aliquot (150 μL) of supernatant was counted in Amersham Aquasol scintillant. Data expressed as percent inhibition with respect to a control (absence of competing compound) incubation.

(*Received in USA* 17 *August* 1995; *accepted* 1 *April* 1996)