Vol. 149, No. 1, 1987 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 30, 1987 Pages 79-85

CANTHARIDIN POISONING ASSOCIATED WITH SPECIFIC BINDING SITE IN LIVER

Michael J. Graziano[1], Andrew L. Waterhouse[2], and John E. Casida[3]

Pesticide Chemistry and Toxicology Laboratory,
Department of Entomological Sciences, University of California,
Berkeley, California 94720

Received October 14, 1987

Cantharidin, the potent vesicant and toxicant of blister beetles, was prepared as a radioligand to probe its mechanism of action. [^{3}H]Cantharidin interacts in a saturable and specific manner with a binding site in mouse liver cytosol with apparent K_d and B_{max} values of 30 nM and 1.8 pmol/mg protein, respectively. Comparisons of cantharidic acid, the related herbicide endothal, and 20 other oxabicycloheptane-dicarboxylic acids show that their potency as inhibitors of [^{3}H]cantharidin binding is closely correlated with their intraperitoneal toxicity to mice. This binding site is also inhibited in vivo by toxic doses of cantharidin. The [^{3}H]cantharidin binding site in mouse liver cytosol therefore represents, or serves as a model for, the site of toxic action of cantharidin and structurally-related compounds.

Cantharidin is the active ingredient of "Spanish fly", a counterirritant and purported aphrodisiac consisting of dried blister beetles, and as such it has been the cause of frequent human poisonings (1-3). Ingestion of only a few beetles provides enough cantharidin to be lethal to an animal as large as a horse (4-6). It is a severe irritant to epithelial linings, leading on oral administration to ulceration, hemorrhaging, and necrosis of the gastrointestinal and urinary tracts (2, 3, 4) and on dermal contact to blister formation and a separation of cells known as acantholysis (7, 8). Cantharidin poisoning also results in congestion and edema of the liver (3, 9, 10), which histologically is very similar to acantholysis of skin tissue (7, 8). For this reason, the liver is used as a model target organ in studies on its mode of action (8-13). Although it is proposed that cantharidin interferes with mitochondrial respiration (12, 13) or activates an acantholytic factor (14), its mechanism of toxicity remains unknown.

1 Present address: Department of Pathology and Experimental Toxicology, Parke-Davis Pharmaceutical Research Division, Warner-Lambert Company, Ann Arbor, MI 48105.

2 Present address: Department of Chemistry, Tulane University, New Orleans, LA 70118.

3 To whom correspondence should be addressed.

0006-291X/87 $1.50

The present investigation uses the radioligand approach in recognizing a specific binding site in mouse liver. Based upon structure-activity relationships with several analogs, as well as in vivo binding data, this binding site appears to be related to or serve as a model for the site of toxic action of cantharidin.

EXPERIMENTAL PROCEDURES

Chemicals. [5,6-^{3}H]Cantharidin (14 Ci/mmol, > 98% radiochemical purity) was synthesized by a two-step reduction sequence based on the Dauben procedure (15) as shown in Fig. 1. Briefly, the dehydrothiocantharidin precursor in ethyl acetate was reduced with tritium gas at one atmosphere using a palladium/carbon catalyst and a reaction time of 4 hr at 25°C. The [5,6-^{3}H]-intermediate in ethanol was then desulfurated with W-2 Raney nickel on refluxing for 3 hr under nitrogen. [^{3}H]Cantharidin was purified by high pressure liquid chromatography on a silica column with 5% acetonitrile in chloroform. Its structure was confirmed as the 4-nitrobenzyloxyimide derivative by thin-layer cochromatography (silica gel, chloroform, Rf 0.25) and mass spectrometry (16). The specific activity of [^{3}H]cantharidin was verified by dilution assays with unlabeled cantharidin (17). The cantharidin analogs utilized were synthesized and characterized as previously described (18).

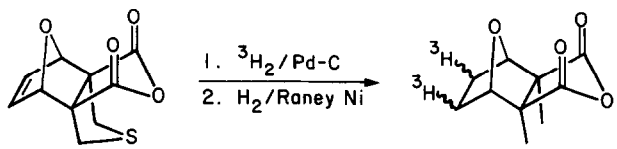

Fig. 1. Synthesis of [5,6-^{3}H]cantharidin.

Preparation of subcellular fractions. Fresh livers from male Swiss-Webster mice were homogenized at 20% (w/v) in ice-cold 20 mM tris-maleate buffer (pH 7.4). The homogenate was subjected to differential centrifugation as follows: 15 min at 1,200 x g, 20 min at 15,000 x g, 20 min at 30,000 x g, and 60 min at 105,000 x g. Each pellet was washed by resuspension in fresh buffer and centrifugation a second time. The washed pellets were then resuspended in fresh buffer equivalent to the original sample volume prior to use in the assays, each with 0.5 mg protein. After initial experiments showed that the cytosol (supernatant at 105,000 x g) contained the major portion of the [^{3}H]cantharidin binding activity, this fraction was used in all subsequent assays. To minimize interactions with endogenous components, the cytosol was routinely dialyzed four consecutive times (10-12 hr each) against 50 volumes of ice-cold buffer. After dialysis, aliquots were stored frozen at -70 °C. Protein was determined by the method of Bradford (19).

Binding assays. The standard binding assay consisted of 0.5 mg cytosolic protein and 5 nM [^{3}H]cantharidin in 1 ml of 20 mM tris-maleate buffer (pH 7.4). For the saturation studies, increasing concentrations of unlabeled cantharidin were added to the incubation mixtures. The samples were incubated at 37°C for 90 min with slow shaking. They were then simultaneously filtered under vacuum through Whatman GF/C glass fiber filters presoaked with 0.3% polyethylenimine (PEI) (20) and rapidly rinsed with buffer (2 x 5 ml) using a Brandel Cell Harvester. Radioactivity retained on the filters was quantitated by liquid scintillation counting. The filtration procedure effectively removed all unbound [^{3}H]cantharidin since additional washes did not alter the amount of radioactivity remaining on the filters. Nonspecific binding was determined by including 10 μM unlabeled cantharidin in the incubation mixture. Higher

concentrations (up to 100 μM) were ineffective at further reducing nonspecific binding indicating that complete saturation of the specific binding sites was achieved. Specific binding is defined as the difference between total binding (with [^{3}H]cantharidin only) and nonspecific binding. Specific binding was corrected for the different specific activities of [^{3}H]cantharidin in the saturation assays. B_{max} (the maximum number of binding sites) and K_d (the binding dissociation constant) were determined by linear regression analysis after Scatchard transformation of the binding data.

Inhibition assays. Analogs were added as solutions (10 μl) in water or acetone to the incubation mixtures and assayed under standard binding conditions. At least four concentrations of each derivative were used giving between 5 and 95% inhibition. The median inhibitory concentrations (IC_{50} values) were determined by linear regression analysis of the binding data from two experiments each run in duplicate.

In vivo binding. For the determination of in vivo binding, mice were given an intraperitoneal (ip) injection of cantharidin in methoxytriglycol at 0, 1, 3, and 10 mg/kg. Thirty min after dosing, the livers were removed and the cytosol prepared as previously described except without dialysis. Prior to assay with [^{3}H]cantharidin, the cytosol was chromatographed on Sephadex G25-150 to remove unbound cantharidin. Preliminary experiments showed that this procedure did not dissociate the cantharidin-binding site complex formed in vivo.

Toxicity assays. Median lethal doses (LD_{50} values) were determined 72 hr after ip administration of the compounds in saline or methoxytriglycol to male Swiss-Webster mice (18).

RESULTS AND DISCUSSION

Mouse liver components involved in specific binding of [^{3}H]cantharidin are localized predominantly in the cytosolic fraction (Table I). There is no

TABLE I

Specific Binding of [^{3}H]Cantharidin to Subcellular Fractions of Mouse Liver

Fraction[a]	cpm/ mg Protein[b]
1,200 x g pellet	239
15,000 x g pellet	431
30,000 x g pellet	113
105,000 x g pellet	64
105,000 x g supernatant[c]	2,674

[a] Subcellular fractions isolated by consecutive differential centrifugation of a 20% w/v homogenate (20 mM tris-maleate buffer, pH 7.4) of fresh mouse liver.

[b] Results are the means of two separate experiments. Assays were performed with 5 nM [^{3}H]cantharidin and 0.5 mg protein/ml (nondialyzed fractions) under standard conditions described in the text.

[c] Glass fiber filters were presoaked in 0.3% PEI for determination of [^{3}H]cantharidin binding to cytosolic fraction.

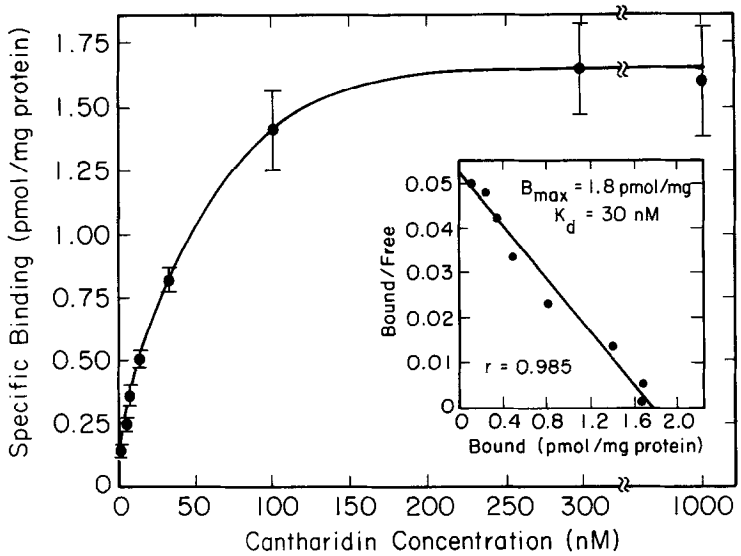

Fig. 2. Specific binding of [^{3}H]cantharidin to dialyzed mouse liver cytosol shown as a saturation isotherm and a Scatchard plot (insert). Assays were performed with 5 nM [^{3}H]cantharidin and 0.5 mg protein/ml at 37°C for 90 min. Nonspecific binding was determined by the addition of unlabeled cantharidin (10 μM final concentration). Data points represent the means and standard errors of three experiments each run in duplicate.

appreciable specific binding to any of the membrane fractions. In order to detect the soluble ligand-binding site complexes, it is necessary to presoak the glass fiber filters in 0.3% PEI. Specific binding of [^{3}H]cantharidin to sites in the dialyzed cytosol is saturable with an estimated K_d of 30 nM and a B_{max} of 1.8 pmol/mg protein (Fig. 2). Nonspecific binding is less than 15% of total binding after correction for a 5% filter blank. The binding site is thermolabile, undergoing 64% loss of activity when held 10 min at 55°C.

Association of [^{3}H]cantharidin with its binding site reaches equilibrium at 90 min. Dissociation of the equilibrated [^{3}H]cantharidin-binding site complex is slow with a half-time of 12 hr for displacement by 10 μM cantharidin. Covalent derivatization is not involved since addition of trichloroacetic acid (10% final concentration) to the incubation mixture after equilibration results in immediate release of all bound radioactivity. These results suggest that cantharidin binding alters the conformation of the receptor protein resulting in occlusion of the radioligand.

The toxicological relevance of the [^{3}H]cantharidin binding site was established by structure-activity assays with various oxabicycloheptane-dicarboxylic acids synthesized as previously described (18). Preliminary assays showed no difference in the ability of cantharidin or cantharidic acid to inhibit [^{3}H]cantharidin binding. There is an excellent correlation (r=0.962) for the eight active dicarboxylic acids between binding affinity (as determined by IC_{50} value) and acute toxicity to mice (Fig. 3). The relationship between binding

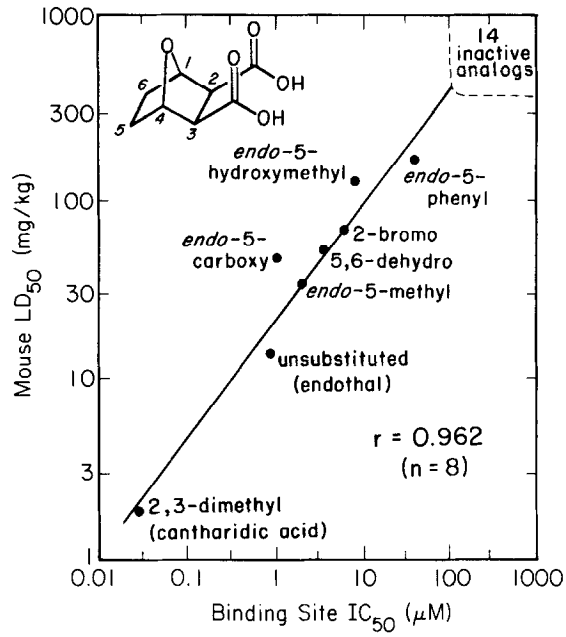

Fig. 3. Correlation for 22 oxabicycloheptane-dicarboxylic acids between potency as inhibitors of [^{3}H]cantharidin binding and toxicity to mice. The generic structure shown is that of the herbicide endothal (exo,exo configuration). Candidate inhibitors were added as solutions in water or acetone. The standard binding assay conditions are given in Fig. 2. LD_{50} values are by ip administration. The inactive analogs (IC_{50} values of ≥100 μM and LD_{50} values of >400 mg/kg) were the endo,exo and endo,endo isomers of endothal, endo,endo-1,4-dimethylendothal, and 11 derivatives of endothal (1-methyl; 1-ethyl; 1,4-dimethyl; exo-5,6-epoxy; exo,exo-5,6-dihydroxy; exo,exo-5,6-methylenedioxy; endo,exo-5,6-dibromo; endo,exo-5,6-dichloro; exo,exo-5,6-dibromo; endo,endo-5,6-dimethyl; 2,3-dehydro).

and toxicity is further strengthened by the observation that all 14 analogs which are ineffective at inhibiting [^{3}H]cantharidin binding are also nontoxic to mice. There is a very precise structural requirement for optimal biological activity. The exo,exo-dicarboxylic acid configuration is essential. 2,3-Dimethyl substitution (cantharidic acid) greatly enhances both binding affinity and acute toxicity, whereas 1,4- or 5,6-dimethyl substitution completely eliminates all activity. It is interesting to note that aside from cantharidic acid, endothal, the unsubstituted derivative and a widely-used herbicide (21), is the most toxic compound to mice and is also the most potent at inhibiting [^{3}H]cantharidin binding. Endothal is corrosive to the skin and epithelial linings of the gastrointestinal tract (22,23) and its biochemical alterations in liver are also the same as those of cantharidic acid (24). The structure-activity data, therefore, strongly suggest that the mechanism of cantharidic acid and endothal toxicity is directly related to their interaction with a cytosolic target protein. Two other observations support this proposal. First, doses of cantharidin giving moderate to severe poisoning signs 30 min after ip administration, i.e, 3-10 mg/kg, cause 50-70% in vivo inhibition of

the liver cytosol binding site. Second, similar cytosolic sites for specific [^{3}H]cantharidin binding are present in brain, heart, kidney, skin, spleen and stomach.

The binding site protein may be a latent cytosolic protease which is activated through a conformational change on binding of cantharidin or structurally-related analogs. This hypothesis for the biochemical mechanism of cantharidin toxicity is based on the present study, the properties of the cantharidin-induced acantholytic factor (14), and recent reports on latent cytosolic proteases which, when activated, can lead to cellular disruption and toxicity (25, 26). In support of this proposal, cantharidin-binding proteins are present in all tissues examined, cantharidin-induced conformational changes are evident in the binding proteins from at least the liver and brain, and the action of cantharidin apparently involves disruption of cell-cell contact.

ACKNOWLEDGEMENTS

This research was supported in part by NIH Grant P01 ES00049. The authors thank W.G. Dauben of the Department of Chemistry (University of California, Berkeley) and M. Matsuzawa of this Laboratory for providing cantharidin analogs, I.N. Pessah of this Laboratory for technical advice in the binding studies, and H. Morimoto of the National Tritium Labeling Facility (Lawrence Berkeley Laboratory, NIH Grant IP 41-RR01237) for assistance in radiosynthesis.

REFERENCES

1. Cavill, G.W.K., and Clark, D.V. (1971) In Naturally Occurring Insecticides (M. Jacobson, and D.G. Crosby, Eds.) pp. 271- 305. Marcel Dekker, NY.
2. Nickolls, L.C., and Teare, D. (1954) Brit. Med. J. 2, 1384-1386.
3. Oaks, W.W., DiTunno, J. F., Magnani, T., Levy, H.A., and Mills, L.C. (1960) Arch. Intern. Med. 105, 574-582.
4. Beasley, V.R., Wolf, G.A., Fischer, D.C., Ray, A.C., and Edwards, W.C. (1983) J. Amer. Vet. Med. Assoc. 182, 283-284.
5. Ray, A.C., Post, L.O., Hurst, J.M., Edwards, W.C., and Reagor, J. C. (1980) Amer. J. Vet. Res. 41, 932-933.
6. Schoeb, T.R., and Panciera, R.J. (1979) Vet. Pathol. 16, 18-31.
7. Stoughton, R.B., and Bagatell, F. (1959) J. Invest. Dermatol. 33, 287-292.
8. Weakley, D. R., and Einbinder, J. M. (1962) J. Invest. Dermatol. 39, 39-45.
9. Opie, E.L. (1912) J. Exptl. Med. 16, 831-849.
10. Bagatell, F.K., Dugan, K., and Wilgram, G.F. (1969) Toxicol. Appl. Pharmacol. 15, 249-261.
11. Allison, J.H., and Williamson, D.H. (1960) Brit. J. Dermatol. 72, 383-402.
12. Decker, R.H. (1963) J. Invest. Dermatol. 42, 465-469.
13. Bagatell, F.K., and Dimitrov K. (1965) Biochem. Pharmacol. 14, 245-254.
14. Bagatell, F.K. (1964) J. Invest. Dermatol. 43, 357-361.
15. Dauben, W.G., Kessel, C.R., and Takemura, K.H. (1980) J. Amer. Chem. Soc. 102, 6893-6894.
16. Ray, A.C., Tamulinas, S.H., and Reagor, J.C. (1979) Amer. J. Vet. Res. 40, 498-504.

17. Akera, T., and Cheng, V.-J.K. (1977) Biochim. Biophys. Acta 470, 412-423.
18. Matsuzawa, M., Graziano, M.J., and Casida, J.E. (1987) J. Agric. Food Chem. 35, 823-829.
19. Bradford, M. (1976) Anal. Biochem. 72, 248-254.
20. Bruns, R.F., Lawson-Wendling, K., and Pugsley, T.A. (1983) Anal. Biochem. 132, 74-81.
21. Carlson, R., Whitaker, R., and Landskov, A. (1978) In Analytical Methods for Pesticides and Plant Growth Regulators (G. Zweig, and J. Sherma, Eds.) Vol. 10, pp. 327-340. Academic Press, NY.
22. Keckemet, O. (1980) Proc. Brit. Crop Protect. Conf.-Weeds 1, 715-722.
23. Allender, W.J. (1983) J. Anal. Toxicol. 7, 79-82.
24. Graziano, M.J., and Casida, J.E. (1987) Toxicol. Lett. 37, 143-148.
25. Nicotera, P., Hartzell, P., Baldi, C., Svensson, S.-A., Bellomo, G., and Orrenius, S. (1986) J. Biol. Chem. 261, 14628-14635.
26. Tanaka, K., Ii, K. Ichihara, A., Waxman, L, and Goldberg, A.L. (1986) J. Biol. Chem. 261, 15197-15203.