ANTINEOPLASTIC AGENTS. 76. THE SEA URCHIN *STRONGYLOCENTROTUS DROEBACHIENSIS*[1]

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ABSTRACT.—An improved procedure based on centrifugation at 37,000 g has been developed for isolating the antineoplastic glycoprotein strongylostatin 1 from *Strongylocentrotus droebachiensis* (Müller). The green sea urchin has also been found to contain a second protein anticancer constituent designated strongylostatin 2. Treatment of the murine P388 lymphocytic leukemia with strongylostatin 2 resulted in 39–42% life extension at a dose of 4.5 mg/kg.

In the marine phylum Echinodermata the subphylum Echinozoa contains the free-living echinoderms without arms such as the nearly eight hundred sea urchins (Echinoidea class). Generally the tube feet and movable spines of these animals are used for locomotion. The ventral side contains the mouth ringed with five teeth (Aristotle's lantern) and the anus is on the upper side. The sea urchins are bottom-living and feed on both plants and animals. The broad ranging (circumpolar) green urchin *Strongylocentrotus droebachiensis* Müller (Echinocea superorder, Strongylocentrotidae family) bears glandular-type (2) pedicellariae venom glands which assist in feeding and defense by immobilizing prey (3).[2] For example, treatment with a solution of zinc sulfate evokes an escape response similar to that seen when the urchin is attacked by the predatory starfish *Marthasterias glacialis*; the globiferous pedicellariae are raised with open jaws (4).

The toxic properties of certain sea urchins were noted by Hippocrates (*cf.*, 5), and the venomous pedicellariae of *Strongylocentrotus* were described in 1899 (6). Except for our investigation (7) which resulted in discovery of the antineoplastic glycoprotein strongylostatin 1 in *S. droebachiensis*, which may also be a constituent of the venom, studies of this species have been confined primarily to the following areas. The gelatinous matrix (the jelly coat which induces the acrosome reaction in urchin sperm) surrounding the *S. droebachiensis* egg was found to contain approximately 20% of a sialoprotein and 80% of a fucose sulfate polysaccharide (8,9). The sperm chromatin proteins (10,11) and nucleotides (12) as well as the embryo ciliary proteins (13) have also received preliminary investigation. Recently the egg jelly coat sialoprotein sialic acids of the sea urchin *Pseudocentrotus depressus* (Okayama) have been found to be *N*-glycoloylneuraminic acid and its 9-O-acetyl-derivative (14).

While strongylostatin 1 was found to produce 35–53% life extension in the National Cancer Institute's (NCI) P388 murine lymphocytic leukemia (PS *in vivo* system) and seemed to reflect most of the observed anticancer activity, we subsequently obtained evidence for the existence of a second antineoplastic glycoprotein herein named strongylostatin 2. A description of the isolation of this substance and an alternate rapid method for obtaining strongylostatin 1 now follows. In contrast to previous collections of *S. droebachiensis* where antineoplastic activity was exhibited by a water extract only, the 2-propanol shipping solution of a 1977 recollection was found to show PS inhibition. The difference was accounted for by an increase in the amount of sea water included in the shipping solution. After removal of the solvent, the crude 2-propanol-water extract was dissolved in water and a portion of the inactive material was eliminated by centrifugation at 2500 g. Strongylostatin 1 was obtained by a procedure

[1]Refer to reference (1) for part 75.

[2]A pedicellariae sting is sufficient to kill a small eel (medulla bite) or snail and stop a frog's heart (Ref. 3).

based on centrifugation at 37,000 g and was thereby easily separated from the much lower molecular weight stronglyostatin 2. Since strongylostatin 1 is large enough to sediment at this gravitational force, the centrifugation method provided a rapid and convenient large-scale isolation procedure. Strongylostatin 1 obtained by the earlier (7) more complicated macroreticular resin→gel permeation method was also found to sediment at 37,000 g.

Strongylostatin 2 was isolated (by the PS *in vivo* system bioassay procedures) following the 37,000 g centrifugation step by fractionation of the supernatant on Sephacryl S-200 and Sepharose 4B; it was found to be a glycoprotein with a unit/subunit molecular weight of at least 65,000. An amino acid analysis showed Gly to be most prominent, and the carbohydrate portion was estimated (15) to represent 22% of the glycoprotein. The marked differences in the amino acid compositions of the strongylostatins eliminate the possibility that strongylostatin 1 is an aggregate of the 2.

In related investigations of the hammerhead shark *Sphyrna lewini* (16) and the Caribbean sea urchin *Lytechinus variegatus* (17), the antineoplastic components were also found to be glycoproteins. Such proteins are bonded to carbohydrate (ranging from about 3.5–81% by weight) units (generally D-xylose, L-fucose, D-glucose, D-galactose, D-mannose, N-acetyl-D-glucosamine, N-acetyl-D-galactosamine, 18). The terminating unit may be one of eight of the nine carbon sialic acids (*e.g.*, 5-acetamido-3,5-dideoxy-D-glycero-α-D-galacto-nonulopyranosonic acid, 19,20).[3] Even relatively small glycoprotein enzymes of the deoxyribonuclease A-D type (mol. wt. 31,000) serve important functions in cell growth and division.[4] Other glycoproteins transport hormones (or are hormones such as LH and FSH) and store hormones (thyroglobulin), ions (calcium II and iron III), and vitamins (B_{12}) or even provide the viscous mucus of gastric mucosa and salivary glands. An anionic glycoprotein with a high content of sialic acid has been found to be released by a cell line of human melanoma. The level of sialic acids in cancer patients are generally elevated, especially in patients with metastatic adenocarcinoma and squamous cell carcinoma (22).

Strongylostatin 2 produced a 39–42% life extension in the PS *in vivo* system (23) at a dose of 4–4.5 mg/kg and will be evaluated further in other NCI exploratory tumor systems.

EXPERIMENTAL

Collection and extraction.—The well known green sea urchin *Strongylocentrotus droebachiensis* was recollected (7) near Canso, Nova Scotia, in the summer of 1977. The animals were preserved and shipped in 2-propanol. The shipping solution was drained from the specimens, and the animals (about 250 kg net weight) were crushed and extracted (48 hr with each solvent) successively with hot ethanol and hot water. The shipping 2-propanol solution (347.8 g extract) and the ethanol extract (503.0 g) were evaporated to dryness, and the aqueous extract (278.5 g) was lyophilized. Samples of the dry extracts were submitted for PS *in vivo* evaluation (23). Only the 2-propanol shipping solution displayed activity (PS T/C 153 at 5 mg/kg).

General methods.—Gel permeation chromatographic procedures were conducted with Sephadex G-50, Sephacryls S-200 and S-300 and Sepharose 4B obtained from Pharmacia Fine Chemicals, AB, Uppsala, Sweden. Water and other solvents were distilled. A Beckman model L-2 ultracentrifuge was used for high speed centrifugation and an International portable refrigerated centrifuge for low speed centrifugation. A Gilson Holochrome spectrophotometer (uv) was employed to monitor column effluent and a Gilson FC-80 microfractionator was used to collect column fractions. The amino acid composition of Strongylostatin 2 was determined on a model 121 Beckman-Spinco amino acid analyzer. Trace metal analyses were obtained on a Jarrell-Ash 3.4 M spectrograph. Other elemental analyses were performed at the Spang Microanalytical Laboratory, Eagle Harbor, Michigan.

Isolation of strongylostatin 1.—A 120 g specimen of the 2-propanol extract was shaken in 1.2 liters of water for 1 hr. Insoluble and very high molecular weight material was removed

[3]When the terminal sialic acid is cleaved and exposes a penultimate galactose unit, this serves as a signal for the liver to remove this glycoprotein from the plasma.

[4]A large (mol. wt. $2x10^6$ in the presence of calcium II ions with dissociation to $2x10^5$ without the metal ions) glycoprotein has been found to be important in aggregation of the marine sponge *Microciona parthena* (21).

by centrifugation at 2500 g for 1 hr. The sediment was washed twice by suspension in 1.2 liters of water and centrifugation at 1,000 g for 15 min. The supernatant aqueous solutions were combined and concentrated (*in vacuo* at 50°) to approximately 800 ml. Crude strongylostatin 1 was separated from the concentrated supernatant fraction by centrifugation at 37,000 g for 1 hr. The sediment (strongylostatin 1) was washed by resuspension in 60 ml of water and centrifuged at 1,000 g for 20 min to remove residual insoluble and very high molecular weight material. The supernatant was centrifuged at 37,000 g for 1 hr to collect the purified strongylostatin 1. The pellet was resuspended in a small quantity of water and lyophilized to yield 3 g (2.5%) of strongylostatin 1 with the same physical and biological properties (PS T/C 153 at 10 mg/kg) as previously found for this high molecular weight glycoprotein.

ISOLATION OF STRONGYLOSTATIN 2.—The aqueous supernatant obtained above (from centrifugation of the crude extract at 37,000 g) was freeze-dried (PS T/C 116 at 16 mg/kg). A sample (1 g) was chromatographed (room temperature) on Sephacryl S-200 (2.6 x 88 cm) in 0.02 M Tris hydrochloride buffer (pH 7.0) containing 0.5 M sodium chloride. Elution of the column effluent was monitored by absorbance at 280 nm; the effluent was collected in 7.5 ml fractions. Five major fractions were obtained: fractions 21–23, 27–31, 45–57, 59–64, and 69–73 were dialyzed against water and lyophilized. The void volume fraction (14.6 mg) was found to be active (PS T/C 125 at 2 mg/kg and 123 at 8 mg/kg). All included fractions were inactive. The chromatographic procedure using Sephacryl S-200 was repeated with a preparative column (5 x 81 cm) and 6–8 g portions of the lyophilized supernatant fraction. Yields (4–5%) of the large-scale active void volume fraction (S-200, V_0) amounted to some 0.30 g.

The resultant material (S-200, V_0) was poorly soluble in water, 0.02 M Tris/HCl-0.5 M NaCl (pH 7.0), 1% acetic acid, 4 M guanidine hydrochloride, and ethanol but dissolved in 0.02 M Tris/HCl (pH 7.0)-1% sodium dodecyl sulphate (SDS). Portions (120 mg) of the S-200, V_0 fraction were chromatographed on Sepharose 4B (2.5 x 100 cm) in 0.02 M Tris/HCl (pH 7.0)-1% SDS. The column effluent absorbance at 280 nm was monitored and collected in 7.0 ml fractions. The three fractions obtained by combining the contents of fractions 17–23, 57–59, 60–66 were dialyzed against water and lyophilized. Residual sodium dodecyl sulphate was removed from each fraction by passage through Sephadex G-50. The major included Sepharose 4B fraction (a tan fluffy powder) proved active (PS T/C 139 at 4.5 mg/kg) and was designated strongylostatin 2. The final yield of the sodium dodecyl sulphate-free material was 0.5–1% of the original lyophilized supernatant. In order to eliminate use of SDS, strongylostatin 2 was also prepared by passage of a S-200, V_0 fraction homogenate through Sepharose 4B (2.5 x 100 cm) in 0.02 M Tris/HCl-0.5 M NaCl (pH 7.0). The major fraction eluted in the void volume and was dialyzed against water and lyophilized. Strongylostatin 2 prepared by this procedure exhibited a PS T/C of 142 at 4 mg/kg and was equivalent to that prepared with SDS, but it did include the minor contaminating void volume fraction. The final yield of strongylostatin 2 prepared by this method was 2% of the original lyophilized supernatant.

Strongylostatin 2 was found to be difficultly soluble in water or 0.02 M Tris/HCl-0.5 M NaCl (pH 7.0), but a clear solution was obtained in 0.02 M Tris/HCl (pH 7.0) containing 0.1–1% SDS. The unit/subunit molecular weight was estimated in 1% SDS on Sephacryl S-300 (1.4 x 63 cm) to be about 65,000 with ferritin (540,000), catalase (240,000), aldolase (158,000) and bovine serum albumin (67,000) as standards chromatographed in 0.02 M Tris-HCl (pH 7.0). Polyacrylamide gel electrophoresis did not prove useful with strongylostatin 2.

The protein content of strongylostatin 2 was estimated to be 10–15% by a modified Lowry method (24) using bovine serum albumin as standard and was consistent (25) with the nitrogen elemental composition. Amino acid analyses of a 6.1 N HCl hydrolysate of strongylostatin 2 (26) showed: Ala 84, Arg 47, Asx 73, Cys 4, Glx 62, Gly 174, His 19, Ile 56, Leu 77, Lys 67, Met 1–2, Phe 40, Pro 74, Ser 61, Thr 74, Tyr 20, Val 67 in amino acid residues/1000. When glucose was used as the standard, the carbohydrate content was estimated by the phenol-sulphuric acid method (15) to be 22%. Metal analyses of strongylostatin 2 confirmed the presence of Na in major amounts (>1.0%, probably from the SDS or buffer), Ca, Mg, Ti in minor amounts (0.01–1.0%), and Al, Cu, Fe in trace amounts (<0.01%).

Anal. Found: C, 46.87; H, 7.12; N, 1.97; F, 0.81.

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