ISOLATION AND CHARACTERIZATION OF STRONGYLOSTATIN 1[1]

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Abstract.—The well-known green sea urchin *Strongylocentrotus drobachiensis* (Müller) has been found to contain a new glycoprotein antineoplastic agent. The glycoprotein was found to produce 35–53% life extension in the P388 murine lymphocytic leukemia system and has been designated strongylostatin 1. Because of the toxicity of strongylostatin 1 it may also be a constituent of *Strongylocentrotus drobachiensis* venom.

Marine invertebrates do not have a thymus system generating antibodies for immunological-type protection (1). Instead, phagocytosis (by encapsulating leukocytes) assisted by protecting substances formed over an exceedingly long evolutionary period comprise the major defensive mechanisms (2). Such considerations led us some fourteen years ago to begin evaluating marine invertebrates and vertebrates as new sources of potentially useful cancer chemotherapeutic drugs (3). Early in this study, we found the venomous sea urchin (West Indies) *Lytechinus variegatus* (Lamarck) (3, 4) of the Toxopneustidae family to provide extracts with antineoplastic activity. Subsequently we uncovered a number of such Echinoderms affording extracts with confirmed level activity in the U.S. National Cancer Institute's murine lymphocytic leukemia P388 (PS system) (5).

We now report that the ubiquitous north temperate ocean (indeed circumpolar) green sea urchin *Strongylocentrotus drobachiensis* (Müller) of the family Strongylocentrotideae which bears venomous globiferous pedicellariae (4) was found to produce a new glycoprotein antineoplastic agent designated strongylostatin 1. Isolation of strongylostatin 1 by the method now summarized was found most convenient. A water extract of *Strongylocentrotus drobachiensis* from the coast of Nova Scotia was triturated with methanol, and the less soluble fraction was chromatographed on the macroreticular resin XAD–2. The material eluted with water was further separated by a series of gel permeation chromatographic steps with Sephadex G–50 followed by Sephadex G–200. The first fraction eluted from the G–200 column by 0.02 *M* ammonium acetate was further separated with Sepharose 2B in a solution composed of 0.025 *M* tris hydrochloride and 0.1 *M* potassium chloride (pH 7.33) as eluent. The first glycoprotein (6, 7) fraction (approximately 1.6% yield based on the aqueous extract) was found to display the most significant antineoplastic activity (PS, 35–53% life extension at 5–10 mg/kg) and was named strongylostatin 1. The apparent molecular weight (8) of strongylostatin 1 appears to be over forty million. The results of amino acid analyses suggested a minimum protein segment of 546 amino acid units.

Since the PS evaluation system was employed as bioassay during isolation, strongylostatin 1 should represent the major antineoplastic component of *Strongylocentrotus drobachiensis*. So far, the level (*ca.*, 10 mg/kg) of toxicity exhibited by strongylostatin 1 suggests that this substance may also be a component of *Strongylocentrotus drobachiensis* venom (9). Preparations are now being made

[1]The present investigation corresponds to part 54 of the series Antineoplastic Agents: see Ref. 14 for contribution 53.

to evaluate this substance in other National Cancer Institute exploratory tumor systems.

EXPERIMENTAL[2]

COLLECTION AND EXTRACTION.—The initial collection of *Strongylocentrotus drobachiensis* was made by G.R.P. (assisted by Mr. W. E. Pettit and Miss M. S. Pettit) near Canso, Nova Scotia, June 1971. Other specimens of this animal collected (by G.R.P., August and October 1971) off the coasts of Alaska gave less active (T/C 120–124) water extracts. A recollection of *Strongylocentrotus drobachiensis* (completed in June 1973, along the coast of Nova Scotia) was preserved in 2-propanol in ten 1-gal containers. The shipping solution was drained from the specimens and the whole animals were crushed and extracted (48 hr) successively with ethanol and water with a modified Soxhlet apparatus. The aqueous extract was lyophilized and submitted for P388 *in vivo* evaluation (5) and found to display toxicity at 25 mg/kg and activity (T/C 145) at 6.25 mg/kg.

ISOLATION OF STRONGYLOSTATIN 1.—A portion of the aqueous extract (50.2 g) was triturated with methanol (1 liter) for 48 hr. The solid portion (31.0 g, PS T/C 167 at 7.5 mg/kg) was dissolved in water and was further separated as follows. Typically, 31.0 g of aqueous extract (methanol insoluble fraction) in 2 liters of water was placed on a column of XAD–2 (4 liters volume). Elution with water (15 liters) gave the first fraction (PS T/C 148 at 2.5 mg/kg) as 21 g (freeze-dried) of water soluble material. The remaining material eluted from the column with methanol (15 liters) was inactive in the PS system.

A Sephadex K–100 column was packed with G–50 Sephadex (7 liters volume). The active aqueous fraction (9.50 g portion) from the XAD–2 separation was dissolved in water and applied to the column. Elution with 1.8 liters of water (at a flow rate of 400 ml/hr) yielded the void volume material (2.4 g) as the active (PS) fraction. No antineoplastic activity was exhibited by the remaining fractions.

A 0.63 g portion of the active fraction was applied to a Sephadex G–200 column (3 x 75 cm) equilibrated with 0.2 *M* ammonium acetate. The first 180 ml of eluate containing a deep straw colored band was active (T/C 136 at 1 mg/kg in PS) while subsequent fractions were inactive. Sepharose 2B chromatography was employed for final purification.

An analytical Sepharose 2B column (2.5 x 33 cm) was prepared and equilibrated with 0.025 *M* tris hydrochloride buffer (pH 7.46) containing 0.1 *M* potassium chloride and monitored with a differential refractometer. Fractions of 2 ml each were collected. A 10 mg sample of the excluded fraction from Sephadex G–200 was analyzed. Three major peaks were observed at elution volumes of 57 ml, 154 ml and 173 ml. The procedure was repeated with a larger Sepharose 2B column (5 x 69 cm) prepared as above. In a typical separation, 0.6 g of excluded material from either Sephadex G–200 or G–50 was applied to the column and fractions were collected according to the analytical model determination. The overlapping components with elution volumes of 154 and 173 ml (analytical), were not completely separated. The water fractions were concentrated *in vacuo*, desalted with a Bio-fiber 50 beaker and lyophilized. Of the two major fractions obtained from the Sepharose 2B separation, the first was found active (PS T/C 153 at 10 mg/kg and toxic at 10–20 mg/kg) and designated strongylostatin 1. From two such Sepharose 2B separations, 1.2 g of G–200 fraction gave 0.35 g of strongylostatin 1. The anticancer agent strongylostatin 1 was shown (6) to be a glycoprotein (35–38% carbohydrate) and amino acid analyses gave the following results: Ala 65, Arg 17, Asx 67, Glx 47, Gly 63, His 6, Ile 28, Leu 47, Lys 25, Phe 26, Pro 28, Ser 33, Thr 37, Tyr 17, Val 40 for a total of 546 units.

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[2]Sephadex G–50 and G–200 as well as Sepharose 2B were obtained from Pharmacia Fine Chemicals. The Amberlite resin XAD–2 from Rohm and Haas was used for initial separation. A Waters Associates Differential Refractometer R–403 was used to monitor column effluent. The column fractions were desalted using a Bio-fiber 50 device from Bio-Rad Laboratories. A Gilson Electronics Model FC–80 microfractionator was used to collect column fractions.

Electrophoresis was performed on a GE–4 Pharmacia unit employing polyacrylamide gradient gels (PAA4/30, Pharmacia Fine Chemicals) and a sodium dodecyl sulfate (SDS) system (10). The resulting gels were stained for protein with Coomassie Brilliant Blue R. The sample molecular weight/molecular size was too large to enter the gel, and neither the normal nor the SDS techniques could be employed.

The glycoprotein (strongylostatin 1) was analyzed for amino acid composition on a model 121 Beckman-Spinco amino-acid analyzer according to procedures (11–13) that do not provide cysteine and tryptophan content. The carbohydrate content (35–38%) was determined by the orcinol-sulfuric acid method (6). A sample of strongylostatin 1 was analyzed for phosphorus at the Spang Microanalytical Laboratory and was found to contain 1.34% of this element. Metal analyses revealed no significant percentages of such elements.

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