Research on Potential Pharmaceutical Products from Australian Holothurians

(1/12/1994 – 31/5/1995)

David Fairlie, Craig Davis and Michael Whitehouse

NATIONAL SEAFOOD CENTRE REPORT

NSC 92/125.22

FISHERIES
RESEARCH &
DEVELOPMENT
CORPORATION

CONTENTS

1. SUMMARY

This final report details all work conducted under a 5 month pilot study funded by a small grant from the National Seafood Centre in December 1994. During this time we (i) collected and/or prepared dry powders from species of eviscerated holothurians; and (ii) evaluated extracts of the powdered specimens for antiinflammatory, anti-ulcer and hypotensive activities in rats.

We examined powdered sea cucumber samples provided to us by a range of individuals and groups including fishermen, the Queensland Department of Primary Industries, and others interested in Beche-de-mer as a food source.
In general we found through this project that :
(a) species provided to us were not reliably or correctly identified;
(b) samples provided had been processed by unspecified or varied means;
(c) species were harvested from different (often unspecified) geographical locations;
(d) samples gave irreproducible results in our biological assays.

We also collected some 14 identifiable specimens ourselves from local areas (Herron Island and Bribie Island), conducted careful taxonomic identification of species, and utilised a simple post-catch handling method to standardise materials prior to testing in vivo.

In summary, we found that people in the industry are not sourcing/handling/processing sea cucumbers adequately for retention of therapeutic activities even though such methods may be appropriate for the food industry. We identified that control over species collection, handling, identification, processing and evaluation should be the first priority in any efforts to develop a therapeutic-based sea cucumber industry. We have confirmed our previously published studies that some species of sea cucumbers do have potential therapeutic properties.

2. INTRODUCTION

Sea cucumbers have been used for centuries by the Chinese in traditional cooking and are highly regarded as aphrodisiacs and dietary supplements. Worldwide there are some 1200 species of sea cucumbers (Beche-de-mer or trepang, or sea slug) including over 100 species around Australia, but less than 20 species are presently harvested as a food supplement mainly for South East Asian markets. The Australian food product has low value and represents a small industry with less than 300 tonnes wet weight product per annum (85% water) compared with a world trade of more than 12,000 tonnes dry weight product per annum.

In 1994 Australian researchers Fairlie and Whitehouse (now at University of Queensland) published results of some sea cucumbers with activity in rats for the treatment of arthritis, ulcers and high blood pressure (Inflammopharmacology 1994, 2: 411-417). Each of these human pharmaceutical markets is worth many billions of dollars annually and a high-value, low volume product for such markets can be worth $1000s per kilogram. In the case of sea cucumbers, if a safe and active pharmaceutical product could be developed, it may not need to rely on high volume catches of a few selected species of edible sea

cucumbers and could be marketed worldwide instead of just as a food to limited markets in South East Asia.

3. COLLECTION, HANDLING, IDENTIFICATION AND PROCESSING

Specimen Collection

Species of Beche-de-mer were provided by a range of individuals from North Queensland. In general we were not provided with detailed information regarding their specific origin, method of handling/processing and species identification, but were simply given powders to examine. As a result samples that were supposedly "the same" but from different sources usually had widely differing properties. Due to financial limitations and geographical constraints, we were not able to exercise any direct quality control over these collections.

During part of the project we therefore resorted to collecting samples ourselves from local environments (Herron Is and Bribie Is). There is considerable evidence that variations in geographical origin, climatic and environmental conditions can influence the extent of medicinal activities even within the same species. By establishing taxonomic identities ourselves, and controlling methods of handling, we tried to systematically examine and compare sea cucumbers for a variety of species that were identified, treated and powdered in exactly the same manner. Our efforts were compromised by the timeframe of the project and limited resources but did identify various problems.

Species Identification : Taxonomy of Beche-De-Mer

Correct identification of Beche-de-mer species is crucial if some degree of quality control over harvesting is to be maintained. Beche-de-mer belong to one class (Holothuriodea) of the phylum Echinodermata. They are characterised by a pentaradial body plan and a water vascular system. They are restricted to the sea and are common to all latitudes and all depths of the oceans (Cannon, L and Silver, H., 1986). The following characteristics were used in species identification :

1. Size
2. Colour
3. Surface characteristics (form of papillae and their distribution)
4. Texture
5. Calcareous plates in their skin
6. Tentacles
7. Presence or absence of tube feet
8. Presence or absence of Cuvierian tubules
9. Type of small calcareous particles (spicules) in the body wall - this feature is used in distinguishing Holothurians.

Spicule Preparation

From the stored specimen a piece of body wall was cut off for species identification by spicule analysis. The procedure for preparation of the spicules from Beche-de-mer body walls was as follows:

1. Take a small sample (approx. 1 mm^3) into an eppendorf tube
2. Add approximately 1 mL of concentrated Bleach and leave at room temperature for approximately 1 hour
3. Remove the supernatant and discard

4. Add approximately 1 mL of distilled water and centrifuge briefly in the eppendorf centrifuge
5. Remove the supernatant and discard
6. Allow the spicules to air dry
7. Add 100 microlitres of glycerol and water (1:1) and mix thoroughly
8. Add one or two drops to a microscope slide
9. Carefully place a cover slip onto the sample
10. Allow to dry overnight (if necessary)
11. Seal with nail polish
12. View under microscope attached to video camera and a computer
13. Print images of the different spicules observed
14. Store slides flat (not vertical)

Spicule Analysis (Appendix 1).

To *confirm visual* macroscopic *characterisation of Beche-de-mer species*, we utilised a microscopic analysis of spicules. Small samples (1-2 mm^3) of the flesh of each Beche-de-mer specimen was removed and immersed in a solution of bleach overnight. The insoluble material was concentrated by centrifugation and washed twice with distilled water. Spicules were resuspended in glycerol:water (1:1 w/w) and placed on a microscope slide. A coverslip was carefully placed on top and held in place with nail polish. The slide was viewed at 10-40 magnification (see Appendix 1).

Handling & Processing of Specimens

After collection, the animals were identified (as above) and photographed. The probable species name and common name were then recorded. Each was given a code name; date collected and date processed were recorded. A proper description of the animal was recorded. The animal was then measured in water; measured and weighed out of water; gutted and weighed; and freeze-dried and weighed. This sample was then stored for later hammer milling.

To avoid complications relating to the use of patented processing methods, we decided to treat all samples that were obtained directly by us in the following straightforward manner. Sea cucumber specimens (5 of any given species, where possible) were collected, dissected from end to end, and the viscera removed (animals were maintained in salt water aquaria for up to 12 hours, when necessary). The body tissue was frozen and stored at -70 degrees prior to transport to the laboratory. A sample of the body tissue (20-30 grams) was freeze-dried and milled with a laboratory hammer mill into a fine dry powder. The final sieve size was 0.25 mm diameter. Heat generation was minimised in all the processing steps.

Process for Stabilisation

The medicinal properties can be optimised in holothurian extracts if the freshly collected animals are immmediately treated with certain preservative mixes prior to processing to remove, cure and dry body walls. These methods were developed and provisionally patented (M. Whitehouse, June 1994) prior to undertaking this project and are commercial in confidence. Such treatments lead to much more active beche-de-mer extracts. We note that many of the powder samples provided to us in this project were obtained through various heating procedures which can, depending upon the method, inactivate sea cucumbers with respect to their medicinal properties even though such

procedures are effective for developing food products. In view of the patent position, we opted instead not to treat samples other than as described above.

4. METHODS FOR PHARMACOLOGICAL EVALUATION

4.1 Acute Anti-Inflammatory Activity

Female Wistar rats (180-220 g) were injected in each rear paw with 0.6 mg sodium carrageenan dispersed in 0.1mL normal saline (0.15M NaCl) to elicit an acute oedema. This was quantified by determining change in paw thickness, measured with a micrometer screw gauge at 1, 2, 3 and 24 hours after injecting the carrageenan. Samples of resuspended dried Holothurian powder were evaluated at a dose of 300 mg/kg for anti-inflammatory activity action by administration to rats by mouth 45 minutes before carrageenan injection. Each Holothurian extract was assayed for this anti-oedemic activity, given alone and also when given with a prostanoid synergist (0.4 mg/kg).

4.2 Chronic Anti-inflammatory Activity

Female Wistar rats were injected in the tail base with an arthritigenic (Freund's type) adjuvant composed of 1mg of heat-killed dried *Mycobacterium tuberculosis (hominis)* dispersed in 0.1 mL squalane on Day 0. On Day 10, they were assessed for initial signs of arthritis by measuring rear paw and (maximum) tail thickness, noting any inflammation of the forepaws (scaled 0 to 4+) and recording their body weight. Groups of 4 animals were then orally administered once daily with test samples for 4 days (i.e. days 10 through 13 inclusive) and reassessed for signs of arthritis on day 14. Those groups in which signs of arthritis were much reduced (less paw inflammation/weight loss) were then re-evaluated on day 17, the purpose being to assess rebound in arthritic signs after ceasing treatment. Animals showing no such rebound were considered non/poor reactors to the original arthritigen and were therefore eliminated from the assay as likely 'false positives'.

4.3 Anti-ulcer (gastroprotectant) Assay

Rats given the same arthritigen, but not pre-treated with these holothurian extracts, were fasted overnight on day 17 when their polyarthritis was fully expressed and their gastric resistance considerably compromised by this disease "stress". These animals were kept in special open-wire cages for this fast period to ensure minimal coprophagy and to deny access to other consumables (food, bedding). The fasted rats were challenged with ibuprofen (50 mg/kg) to provoke gastric bleeding. Holothurian extracts were given simultaneously as a single oral dose, usually to groups of 3 rats, to assess their gastroprotectant potential. After 2.5h, animals were killed by cervical dislocation, their stomachs excised, cut open and rinsed for macroscopic evaluation of gastric injury. The numbers of point haemorrhagic lesions, their severity and the frequency of incidence of gastric damage in each test group were evaluated and used to prepare a composite Gastric Lesion Index.

4.4 Hypotensive Activity

Female Wistar rats (230-250g) were injected intraperitoneally with filtrates from holothurian extract (150mg/kg) prepared by homogenising with 0.04% Tween-20 and 70% isotonic saline. They were then observed for no longer than 3 hrs before being killed by cervical dislocation. Any signs of flaccidity (hypotension-induced relaxation) or writhing (peritoneal irritation) were recorded. Behaviour of animals after 10 minutes and 110 minutes was monitored by two observers.

5. BIOASSAY RESULTS

5.1 Introduction

Fourteen taxonomically identified species were examined for activities in rats during the course of the program. These were :

#1	Holothuria scabra
#2	Actinopyga miliaris (#2),
#3	H (Microthele) fuscopunctata
#4	Bohadschia argus
#5	H. (Thymiosycia) impatiens
#6	H. (Mertensiothuria) pervicax
#7	Green variant 3
#8	Green variant 2
#9	H. (Microthele) nobilis
#10	Thymiosycia hilla
#11	Stichopus horrens
#12	Stichopus chloronotus (greenfish)
#13	Mertensiothuria leucospilota
#14	Loaves of bread

Four activities were observed :
(1) acute *antiinflammatory activity* in rats - samples producing a short duration of action like non-steroidal antiinflammatory drugs and aspirin;
(2) *antiarthritic activity* in a chronic rat model of arthritis - agents effective in this asssy may be useful therapies for different forms of arthritis in man;
(3) *antiulcer activity* in rats - the particular assay used is commonly employed to identify agents with potential for treating ulcers in man;
(4) *antihypertensive activity* : materials with the potential to reduce blood pressure in rats could potentially be so-used in humans.

5.2 Acute Anti-Inflammatory Activity (Appendix 2)

No species of sea cucumber demonstrated any significant acute antiinflammatory (aspirin-like) activity in suppressing oedema formation over 2 hours. When given with a synergist (data not shown), some species did manifest significant (>35%) activity. These were species # 2, 3 and 11. Neither C-Care nor Seatone, from commercially available samples sold in Januray 1995, showed any statistically significant antiinflammatory activity even at 300mg/kg (twice the dose of aspirin).

5.3 Anti-Arthritic Activity (Appendix 3)

Where possible at least 2 specimens of any one species were examined in this assay. Inactive species included #1, 2 and 6. Species 7 (?), 8, 9, 11, 13 appeared to show some activity. Indeterminate findings were recorded with the others; either being positive with a single specimen (3) or mixed positive and negative (12, 14) when 2 specimens of the same species were examined. In the latter case it's probably more realistic to accept the negative evaluation until further confirmation is obtained through further experimentation. Some reference commercial materials = SeaCare prepared from mixed Holothurian and Seatone/Musseltone from *Perna canaliculus* were included for comparison.

5.4 Gastroprotectant Activity (Appendix 4).
Appendix 3 shows a wide range of efficacy of individual Holothurian extracts in protecting the gastric mucosa of fasted disease-stressed (arthritic) rats from the noxious effect of Nurofen^{R} (50 mg/kg). This is a formulation of ibuprofen, an analgesic/antipyretic/anti-inflammatory drug, that is available as an over-the-counter (OTC) formulation from most pharmacies in Australia. Prolonged use can cause a serious side-effect, the production of gastric ulcers. The species found to be consistently active were #9 and #14. Species not showing significant gastroprotectant effects in this assay included 1, 4, 6, 11, 12 and13. Ambiguous results were obtained with different samples of 10. Commercially available (January 1995) Holothurian samples (Sea Care) were virtually ineffective, as was a commercial anti-ulcer drug (Sucralfate^{R}).

5.5 Hypotensive Activity (Appendix 5).
We have previously found that commercially processed edible preparations of holothurians contain a hypotensive or muscle-relaxing agent that rapidly induces a transient but severe flaccidity when injected into rats. Some species of holothurians are known to contain a muscle relaxant. Appendix 4 shows that several species are hypotensive (#1,3, 4,5,6).

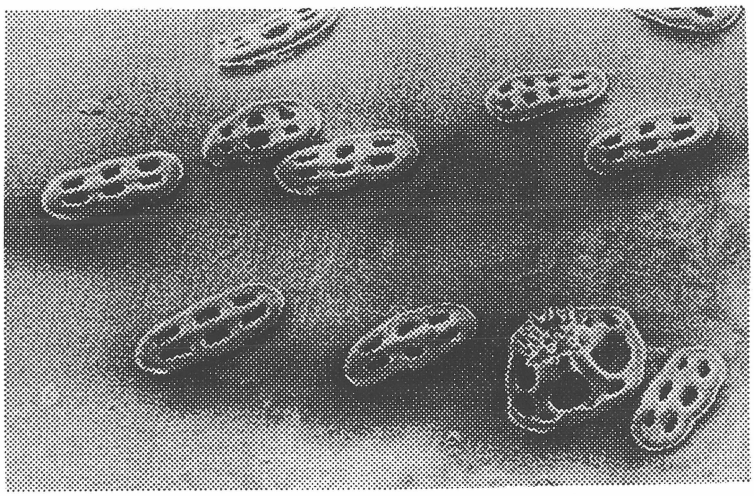
5.6 Bioactivities Summary.
In general the results were somewhat irreproducible for all the bioactivity screens, despite some preliminary observations to the contrary in an interim report for this project provided in March 1995.

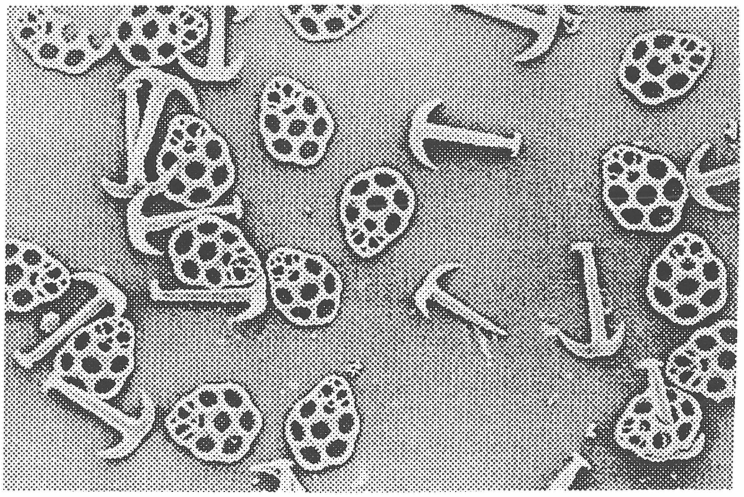
There was no obvious *acute antiinflammatory activity* (Appendix 2) for any of the 14 species, although some activity can be induced if synergising agents are used in combination therapy (as described in Immunopharmacol. 1994, 2: 411-417). Several species showed activity against a *chronic model of inflammation* (Appendix 3), but again when more than 2 preparations were tested in these 3 week long assays we obtained mixed results for some species. Two species showed some significant *anti-ulcer activity*. Some species showed *hypotensive activity* which could be dangerous in consumers with low blood pressure, but might be useful in people with high blood pressure.

Overall the results indicate that there are genuine activities for sea cucumber preparations in animal models of arthritis, ulcers and hypertension. However if these properties are to be properly developed there will need to be considerably more work done to control the sourcing, identification, handling, processing, and evaluation of the materials. Also the greatest benefits and financial returns are likely to be in active extracts enriched in the active ingredients and these may find a market niche.

APPENDIX 1 : Examples of Spicule Analyses.

Microscopic analysis of a selection of spicules showing their distinctive patterns. These patterns are characteristic features that aid in taxonomic identification of species of sea cucumbers.





APPENDIX 2. Inhibition of Carrageenan Paw Oedema in Rats at 300mg/kg Holothurian species given orally.

Species/sample		% Inhibition after : 1 hour	2 hours
#1	A	19	24
	B	(-51)	08
	C	02	(-05)
#2	A	07	18
	O	(-58)	(-14)
#3	A	07	04
#4	A	07	04
#5	A	17	11
#6	A	07	08
	D	05	(-13)
#7	C	(-10)	(-09)
	E	(-03)	16
#8	A	(-06)	07
	D	(-56)	(-02)
#9	C	09	09
	D	07	(-09)
#10	D	08	(-04)
	E	(-58)	07
#11	A	0	10
	E	11	58
#12	C	(-04)	(-09)
	D	14	
#13	B	(-19)	(-05)
	D	(-38)	(-39)
#14	A	05	(-02)
	C	(-07)	(-16)
Reference compounds :			
C-Care 1 (Qld)		19	(-17)
C-Care 2 (Fiji)		**(-07)**	**(-12)**
Seatone® (mussel)		08	(-12)
Aspirin(150 mg/kg)		43	30

APPENDIX 3 : Anti-Arthritic Activity in Female Wistar Rats.

All materials tested at 300mg/kg given once daily on Days 10 through 13 only. Arthritis evaluated on Day 14.

	% inhibition swelling			
CODE No.	Rear paw	Front paw	Change weight	Efficacy* Rating
1A	19	09	(-02)	0
1B	05	33	05	
1C	0	22	(-05)	
2A	(-04)	(-14)	(-04)	U
3A	88	17	09	U
4A	29	38	+05	U
5				U
6A	(-16)	(-33)	(-09)	0
6D	(-09)	(-60)	+06	
7C	82	57	+06	U
7E	33	09	0	
8C	60	30	+06	2+
8E	45	57	+02	
9C	44	86	+13	+
9D	32	33	(-14)	
10				U
11B	32	39	(-01)	2+
11E	75	100	+08	
12A	(-36)	(-30)	+06	U
12D	77	70	+06	
13A	79	90	0	3+
13C	62	80	(-04)	
14A	(-27)	11	+03	U
14B	77	60	+02	
Reference Materials :				
C-Care 1	55	85	+05	2+
Seatone^{R}	69	93	+08	3+
Mussel-tone^{R} (NZ)	18	50	(-06)	+

* 0 = Inactive; + to 3+ = active; U = Undecided, needs further examination (No rating for single test).

APPENDIX 4. Gastroprotection against OTC Ibuprofen (Nurofen^{R}) in arthritic female Wistar rats

Test samples (300 mg/kg) administered p.o. with p.o. Nurofen (50 mg/kg) to animals fasted overnight. Gastric damage was assessed 2.5 hours later.

Sample	Mean No lesions	Gastric lesion index	GP* rating
None	43	56	
None	35	48	
None	27	39	
1A	30	42	0
1B	36	48	0
2A	14	21	2+
3A	03	07	3+
4A	24	36	+
5A	15	27	2+
6A	38	51	0
7D	12	16	2+
8D	18	25	2+
9D	20	27	2+
9B	0	0	4+
9D	07	14	3+
10A	50	62	0
10B	58	71	0
10F	05	09	31
11A	19	32	(+)
12C	19	31	(+)
13D	31	44	0
14C	02	06	3+
14D	03	08	3+
Reference materials			
300 mg/kg			
C.Care 1	23	35	+
C.Care 2	42	55	0
S.Tone	20	26	2+
100 mg/kg			
Sucralfate^{R}	43	56	0

*GP rating is 4+ for GLI = 0-5
3+ for GLI = 6-15
2+ for GLI = 16-29
+ for GLI = 31-40
0 for GLI > 41

APPENDIX 5. Hypotensive Activity.

Rats (2 per group) were injected with the filtrate of specimens extracted with saline Tween-20 at doses equivalent to 150 mg/kg of original solids.

Species	Behaviour After :	
	10 minutes	110 minutes
1A	flaccid	flaccid but mobile
3A	?flaccid	recovered fully
4A	rapid paralysis	flaccid but mobile
5A	?flaccid	?still flaccid
8A	normal	flaccid
Sea Care	flaccid	recovered fully