PHARMAC
Pharmaceutical Management Agency

Community Exceptional Circumstances
Panel Co-ordinator
PHARMAC
PO Box 10-254
Wellington 6143
Phone (04) 916 7553
Fax (09) 523 6870 (redirects to Wgtn)
Email ecpanel@pharmac.govt.nz

5th May 2010

Dear Dr

NHI:
Patient:
D.O.B.:
Medication: **lipid oil extract (Lyprinol); 5-hydroxytrytophan**

The Exceptional Circumstances Panel has examined your application for the above named patient for supplies of lipid oil extract (Lyprinol) and 5-hydroxytrytophan. The conclusion they have reached is that this patient's circumstances do not meet the criteria for Community Exceptional Circumstances.

Community Exceptional Circumstances funding is available in those situations where either
1. The disease/condition is rare, or
2. The response to treatment is unusual, or
3. Some other unusual combination of clinical circumstances.

It appears that none of these situations apply. This patient's conditions (psoriatic arthritis and depression) are not rare, and the response to alternative treatments is not unusual. (Where rare and unusual are understood to be single figures nationally). The clinical circumstances described are not sufficiently unusual for it to be appropriate to fund this patient whilst declining all others applying for the same medications.

If you have any additional information which would demonstrate that the above criteria are met please provide it in writing for the Panel to review. If you have any concerns about the process please contact me at the above address.

Yours sincerely

J Watkins

Jayne Watkins
Signed on behalf of:
Exceptional Circumstances Panel

Investing in Health

PHARMAC
Pharmaceutical Management Agency

New Zealand Government

Return completed form to:

Exceptional Circumstances
Panel Co-ordinator
PHARMAC
PO Box 10-254, Wellington

Phone: 04-916-7553
Facsimile: 09-523-6870
Email: ecpanel@pharmac.govt.nz

Application Form for Community Exceptional Circumstances Approval

27 APR 2010
022677

Please refer to information sheet if necessary. Complete ALL relevant details. Please type or print CLEARLY.
For *a renewal* complete this page and sections 7 and 8 only

Patient Details

Last Name:	
First Name:	
Address:	
Gender:	(Male) / Female
Date of Birth:	
NHI No:	

Details of Applying Practitioner

Last Name:	
First Name:	
Address:	
Phone:	
Facsimile:	NZMC#:
Email:	
Are you a GP [✓] or Specialist [ ]?	

Disease/Condition

*attach further information if appropriate, a clinical report is useful, be specific

FOR PSORIATIC ARTHRITIS

Other conditions
Psoriasis
Depression

Medicine/treatment sought:

Complete fully, attach additional information as necessary to cover all strengths required.

Brand Name:	LYPRINOL
Chemical Name:	MARINE LIPID OIL EXTRACT.
Manufacturer:	
Form and Strength:	CAPSULES 50mg.
Dosage to be used:	4 - 6 / day.
Dosage regimen: *(where applicable)*	
Extemporaneously compound?: Yes [ ] No [ ] *(If Yes, attach a full list of ingredients)*	

Note that if this is not completed an approval cannot be issued

Nominated Pharmacy (if approval given from where will supplies be obtained? This will generally NOT be a hospital pharmacy.)

Name:	
Address:	
Phone:	

1. ENTRY CRITERIA

Complete the criteria to which this application applies.

☐ **(a) Rare condition** (rare is considered to be a prevalence of <10 nationally)

What is the prevalence (not incidence) of the condition in NZ?

1/1000 Prevalence of IBS

6/100,000 Incidence

☒ **(b) Reaction to alternative treatment unusual** (unusual is considered to be <10 nationally)

List all treatments trialled, patient response to each treatment and how often this response to this treatment occurs in NZ. (Note that failure to respond to funded treatments is not generally exceptional. In order to obtain funding through Exceptional Circumstances the nature of the response would need to be considered exceptional).

Treatment	Response of this patient	Rarity (how often would you expect this to occur?)
IBUPROFEN	SEVERE DIARRHOEA & ULCERS	
DICLOFENAC		

☐ **(c) Unusual combination of clinical circumstance applies**

Describe the unusual combination of clinical circumstances and how often this combination occurs in NZ. (Note that end of spectrum treatments are not necessarily approved; patients must be clearly distinct):

I DON'T UNDERSTAND THIS QUESTION

2. CLINICAL BENEFIT AND SUITABILITY

(a) **attach** evidence that it is a safe and efficacious treatment (e.g. full journal articles, **not** just references, conference proceedings or abstracts). Note that a higher degree of proof will be required for unregistered medications or registered medications for non-registered indications.

(b) Is the pharmaceutical registered for this indication in NZ? Yes ☐ No ☑
BUT IT IS REGISTERED IN AUSTRALIA (ARTIST L124441)

If not, has patient consent been obtained for this use as a non-registered medicine ? Yes ☑ No ☐

(c) Attach specialist opinion (if available) or provide contact details of the specialist the patient has seen and who can be contacted by the EC Panel.

– Rheumatologist –

Name of specialist: _____

Address: _____ + OPD letter.

(Note: the Exceptional Circumstances Panel reserves the right to seek any appropriate opinion)

3. OTHER MEDICATIONS

Provide a full list of treatments for this condition that have been tried or considered.

Pharmaceutical	Unsuitable due to:
Paracetol	– does not work
Ibuprofen	– diarrhoea
Diclofenac	– diarrhoea

Please list any other relevant medications that the patient is currently taking:

NIL

4. OTHER ISSUES

Is there any other relevant information that should be considered?

Pt has a BMI of 17 and any reduction to medication like diuretics would put them at risk

5. ATTACHMENTS

Please attach any additional information which may help the Panel in assessing this application, such as relevant clinic letters, supporting references, lab results, hospital admissions record/s, management plan, and any other information which may be relevant. Please list in the table below the information which you are attaching to this application:

Additional information which is attached to this application (to be completed by applicant):

1.	Hypromol
2.	letter from Dr
3.	Completed Medicine listing in Australia
4.	Pain Control and asthma regarding effects of hypromol
5.	Efficacy + tolerability	trusted hypromol already B

(Please continue this list on an additional page if there is more information than the space provided here.) on inflammatory

(6) Novel alternative source ω-3 PUFAs from NZ, Rheumatoid disorders

(7) Anti cyclooxygenase effects | mussels | lipid extract from NZ green lipped

6. COST ESTIMATE

(As this is an application for funding a cost estimate *must* be included. Failure to give a cost estimate may delay processing of the application. Note that applications in excess of $15,000 for the duration of treatment may undergo a cost utility analysis and will require PHARMAC approval).

Cost per year (quoted by nominated pharmacy, based on dosage requested. Cost must be COST BRAND SOURCE without mark-ups or dispensing fees)	$917.17
Anticipated duration of requested treatment: *(Note that approval will generally be given for only 1 year, renewal would then have to be sought)*	ONE YEAR.

7. RENEWAL *(COMPLETE FOR RENEWALS ONLY)*

If this is an application for renewal please attach the following:

1. a **full report** including details of the patient's clinical progress, the continuing need for the medication and the short and long term future management of this patient.
2. append any relevant and **recent specialist review.**
3. append any **relevant investigations** eg laboratory tests, radiology.

8. SIGNATURES

Signature of Medical Practitioner: _____

Date of Request: 15/4/10

9. PATIENT CONSENT

Patient details

Last Name
First Name

CONSENT BY PATIENT

For the purposes of this application form I consent to:

information concerning my medical conditions being given to the Exceptional Circumstances Panel *(and if required, to PHARMAC); and*

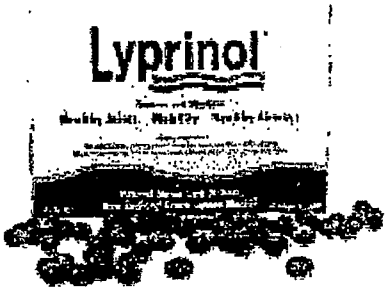
the Exceptional Circumstances Panel seeking further information from medical care providers or seeking further medical opinion as may be necessary for the consideration of my application.

Signed: _____ **Date:** 15.4.10

LYPRINOL®

...NZ's "miracle from the sea"

- Lyprinol® is extracted only from New Zealand's green lipped mussel (Perna canaliculus). It is very concentrated and rich in Omega 3 fatty acids. The tiny capsule is easy for even children to swallow.
- Published international research shows that Lyprinol® provides healthy support for breathing, general mobility, joint movement and overall suppleness.
- Consumers of Lyprinol® are confident that every capsule is effective and full of goodness. The patented extraction process concentrates, stabilises and protects the Polyunsaturated fatty acids (PUFAS), which are the active ingredients, as a pure lipid oil.
- The many Omega 3 fatty acids in Lyprinol® provide powerful support for a range of other essential health functions eg: cardiac, cognitive, digestive, skin, emotional and bowel wellbeing.
- Lyprinol® helps combat the negative effects of trans-fatty acids in the modern diet.
- Everyone is aware of the importance of Omega 3s and polyunsaturated fats in the daily diet. Lyprinol® provides a simple, concentrated and effective way of adding these essential fats to every family's daily intake.
- Lyprinol®, a pure protein free and concentrated lipid oil is packed with the latent health benefits of the green lipped mussel.
- Marine and fish oils are healthy. Lyprinol® is simply better and more effective. Researchers have discovered that, gram for gram, you need far less Lyprinol® for significant health support.
- Sold in 23 countries including: UK, USA, Norway, Australia, France, Germany, Israel, and Korea.



Supplementary to and not a replacement for a balanced diet.

Use only as directed and if symptoms persist consult your Health Professional.

For information Phone:
Apotex NZ Ltd 0800 657 678
Monday to Friday 9am to 5pm.
Or email lyprinol@actrix.co.nz

Distributed by Kenrick Wasland Ltd
PO Box 15 185 Auckland
SRP $49.95

TAPS NA 1-409

TGA THERAPEUTIC GOODS ADMINISTRATION
PO Box 100 Woden ACT 2606 Australia
Telephone: (02) 6232 8444 Facsimile: (02) 6232 8241
ABN 40 939 406 804

Health and Ageing

CERTIFICATE OF MEDICINE LISTING

Listing Name of Therapeutic Goods:
BLACKMORES LYPRINOL

ARTG Listing Number:
AUST L 124441

Commencement Date of Listing:
20 December 2005

CERTIFIED ORIGINAL CERTIFICATE

Sponsor:
BLACKMORES LIMITED

Sponsor Enterprise ID:
10576

The above Medicine is Listed in the Australian Register of Therapeutic Goods subject to the following conditions.

Standard Conditions
The sponsor shall keep records relating to this listed medicine as are necessary to: (a) Expedite recall if necessary of any batch of the listed medicine - (b) Identify the manufacturer(s) of each batch of the listed medicine. Where any part of or step in manufacture in Australia of the listed medicine is sub-contracted to a third party who is not the sponsor - copies of relevant Good Manufacturing Practice agreements relation to such manufacture shall be kept.

The sponsor shall retain records of the distribution of the listed medicine for a period of five years and shall provide the records or copies of the records to the Director - Non-prescription Medicines Branch - Therapeutic Goods Administration - upon the director's request.

The sponsor of the listed medicine must not - by any means - intentionally or recklessly advertise the medicine for an indication other than those accepted in relation to the inclusion of the medicine in the Register.

All reports of adverse reactions or similar experiences associated with the use or administration of the listed medicine shall be notified to the Director - Adverse Drug Reactions Unit - Therapeutic Goods Administration - as soon as practicable after the sponsor of the goods becomes aware of those reports. Sponsors of listed medicines

must retain records of such reports for a period of not less than 18 months from the day the Director - Adverse Drug Reactions Unit is notified of the report or reports.

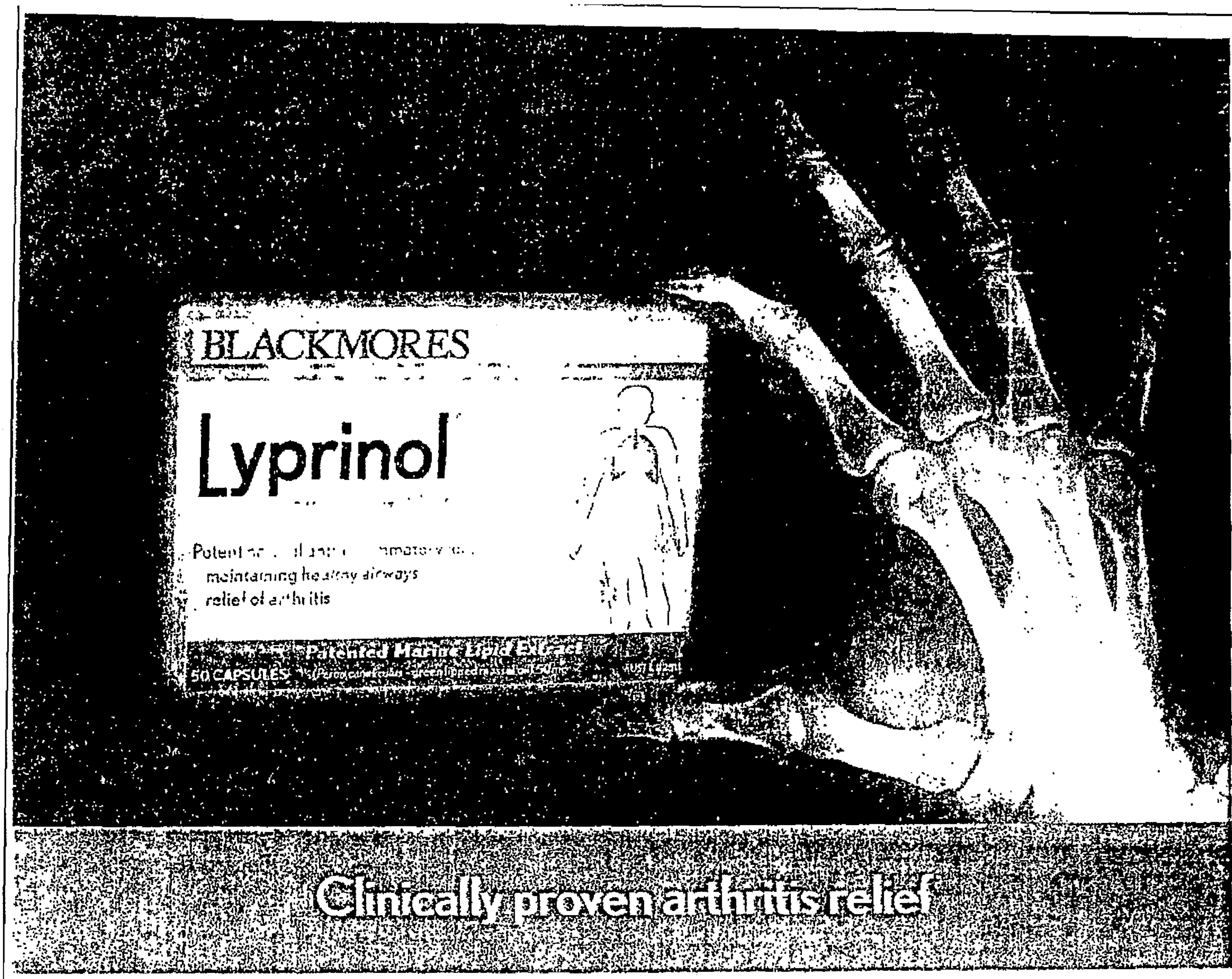
The sponsor shall not supply the listed medicine after the expiry date of the goods.

Where a listed medicine is distributed overseas as well as in Australia - product recall or any other regulatory action taken in relation to the medicine outside Australia which has or may have relevance to the quality - safety or efficacy of the goods distributed in Australia - must be notified to the Director - Non-prescription Medicines Branch - Therapeutic Goods Administration - immediately the action or information is known to the sponsor.

Colouring agents used in listed medicine for ingestion - other than those listed for export only under section 25 of the Therapeutic Goods Act 1989 - shall be only those included in the list of 'Colourings for Use in Pharmaceuticals for Ingestion' included in the Guidelines for Applicants: Listing Drug Products in the Australian Register of Therapeutic Goods for supply in Australia - July 1995 - as amended from time to time.

Export Alias Name

CERTIFIED ORIGINAL CERTIFICATE



Medical researchers, since 1980, have established that New Zealand's green-lipped mussel *(Perna canaliculus)* is well tolerated and an effective way to relieve the symptoms of arthritis and other inflammatory disorders[1].

Blackmores Lyprinol®, is a lipid extract of New Zealand's green-lipped mussel which has undergone a patented extraction process. Clinical studies demonstrated that Lyprinol® reduced joint tenderness, morning stiffness and improved joint function in rheumatoid arthritis and osteoarthritis without adverse effects[2,3,4].

QUALITY ASSURED

Blackmores is dedicated to helping Australians find more natural solutions to today's health issue and has been for over 60 years. Our products are made to high standards using quality vitamins, herbs and minerals. We are committed to providing health professionals with up-to-date reliable information on all aspects of natural health. If you have any questions call our toll-free **Advisory Service on 1800 803 760** or visit our online naturopath at **www.blackmores.com.au**

BLACKMORES
www.blackmores.com.au

REFERENCES. 1. [illegible] 2000;32(7):272-8. 2. [illegible] 3. [illegible] 2002;35(2):[illegible] 4. [illegible]

For more information visit www.blackmores.com.au/lyprinol

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Original Article

Pain Controlling and Cytokine-regulating Effects of Lyprinol, a Lipid Extract of *Perna Canaliculus*, in a Rat Adjuvant-induced Arthritis Model

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[1]State Key Laboratory of Chinese Medicine and Molecular Pharmacology, Shenzhen and Department of Applied Biology and Chemical Technology and [2]Department of Health Technology and Informatics, Hong Kong Polytechnic University, Hung Hom, Kowloon, Hong Kong SAR, China

Using an adjuvant-induced arthritis rat model, we investigated the effects of a lipid extract of *Perna canaliculus* (Lyprinol®) on pain. Radiological examinations, as well as levels of pro- and anti-inflammatory (AI) cytokines, were measured aiming to provide independent objective data to the pain controlling investigation. We confirmed the ability of Lyprinol® to control pain at the initial phase of its administration; with similar efficacy to that observed with Naproxen. The pain scores slowly increased again in the group of rats treated with Lyprinol® after day 9–14. The Naproxen-treated rats remained pain-free while treated. Both Naproxen and Lyprinol® decreased the levels of the pro-inflammatory cytokines TNF-α and IFN-γ, and increased that of IL-10. Extra-virgin olive oil was ineffective on cytokine secretion. Rats treated with Lyprinol® were apparently cured after 1 year. This study confirms the AI efficacy of this lipid extract of *P. canaliculus*, its initial analgesic effect, its perfect tolerance and its long-term healing properties.

Keywords: adjuvant-induced arthritis – inflammatory cytokines – Lyprinol® – NZGLM, pain score

Introduction

Lipid Extract of New Zealand Green-lipped Mussel (Lyprinol®)

A lipid-rich and stabilized extract of New Zealand green-lipped mussel *Perna canaliculus* powder (NZGLM, Lyprinol®), has been shown to have significant anti-inflammatory (AI) activity when given to animals and humans (1–11). This article contributes to the emerging literature on bioprospecting (12). This approach seeks to emphasize for complementary and alternative medicine (CAM) the utility of products derived from animals in treating certain diseases (13–17). Lyprinol® is a mixture of five main lipid classes including sterol esters, triglycerides, free fatty acids, sterols and polar lipids (18,19). There are 90 component fatty acids in Lyprinol® (18,19). Omega-3 polyunsaturated fatty acids are one of the most abundant PUFAs. Eicosapentaenoic acid (20 carbons and 5 double bonds, shorthand = 20 : 5 omega-3) (EPA) and docosahexaenoic acid (22 : 6 omega-3) (DHA) are the major omega-3 PUFAs present in Lyprinol.

Anti-inflammatory Effects

Although the exact active ingredients that brought about this AI effect are unknown, Whitehouse *et al.* (3) reported that Wistar and Dark Agouti rats treated p.o. with this lipid extract did not develop adjuvant-induced polyarthritis or collagen (II)-induced auto-allergic

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arthritis. This was achieved with doses inferior to the ones of NSAIDs, and 200 times lower than other seed or fish oils (3). Further, omega-3 PUFA subfractions of this lipid extract inhibited LTB_4 biosynthesis by neutrophils *in vitro*, and PGE_2 production by activated macrophages (20). Subfractions of this extract containing natural antioxidants (e.g. carotenoids) also exhibited AI activity (5). In contrast to NSAIDs, Lyprinol® is non-gastro-toxic in disease-stressed rats at 300 mg kg^{-1} p.o. (3), and does not affect platelet aggregation in both humans and rats (3).

Clinical studies, either controlled or randomized, have demonstrated that Lyprinol® has highly significant AI activity in patients with osteoarthritis (OA) (4,7,8,21), asthma (22) and other inflammatory conditions (10). Therefore, it seems that Lyprinol® is a reproducible, stable source of bioactive lipids with much greater potency than plant/marine oils currently used as nutritional supplements to ameliorate signs of inflammation (3,10,23). More importantly, in humans and animal subjects taking Lyprinol®, there are no reported side-effects, even at doses up to 2500 mg per day in patients.

Given the AI activities of Lyprinol®, we were interested to know if these AI effects could be translated into pain-controlling effects. We conducted a series of experiments to understand the AI mechanism of action of this lipid extract. This article reports our findings on the pain scores and the accepted causes of pain in rats subjected to severe arthritis induced by adjuvant. In comparison to extra-virgin olive oil (vehicle/negative control) and to the NSAID Naproxen (positive control), we confirmed the pain-controlling ability of Lyprinol® at the initial phase of its administration. This ability is similar to the one observed with Naproxen. Cytokine levels in/from splenocytes of these animals, as well as radiological examination of the affected joints, were also measured to bring objective confirmation to the pain controlling investigation.

Methods

Chemicals

Unless stated otherwise, all chemicals were purchased from Sigma (St Louis, MO, USA). All chemicals were at least of AR grade. Organic solvents used were at least of HPLC grade.

Induction of Inflammation in Sprague-Dawley Rats

Four groups of six 6-week-old male Sprague-Dawley (SD) rats (normal group, adjuvant-induced arthritis (AIA) group, AIA + Naproxen group and AIA + Lyprinol group) and two groups of three 6-week old SD rats (AIA group and AIA + long-term Lyprinol feeding group, respectively) were purchased from the Central Animal Facility (CAF) of Hong Kong Polytechnic University (HKPU). All rats were kept and cared under conditions that fully met requirements of Procedures for the Care of Laboratory Animals or Animals (Control of Experiments) Regulations Chapter 340 of the Hong Kong SAR government. Ethics approval (ASESC No.04/9) was from The Animal Subjects Ethics subcommittee of the HKPU. Arthritis was induced in anesthetized rats by administration of adjuvant according to a method previously described (24,25). Briefly, on Day 0, each rat was injected into the paw of the right hind limb with 100 µl of Freund's complete adjuvant containing 10 mg ml^{-1} of *Mycobacterium butyricum* (Difco, Livonia, MI, USA). Another six rats without arthritis induction were observed as a normal group.

Products/Drug Tested Fed to the Treatment and Control Groups of Rats

Rats in the Lyprinol® (Pharmalink International Ltd, Burleigh Heads, QLD, Australia) group were fed by oral gavage at a dosage of 25 mg Lyprinol kg^{-1} body weight. Typically, the required amount of Lyprinol was made up with olive oil (Virgin®, Bertolli, Italy) to 300 µl and then force-fed to the rats with a stainless steel stomach tube. Three hundred micro liters of olive oil, and 20 mg kg^{-1} body weight of Naproxen were fed as vehicle and positive control. Normal chow was provided to all rats. Except for the long-term Lyprinol-feeding group, feeding of Lyprinol, olive oil and Naproxen were discontinued after the experimental period of 28 days. For the long-term Lyprinol-feeding group, the required amount of diluted Lyprinol (to a dosage of 25 mg kg^{-1} body weight) was added onto 25 g of dry rat chow. These chows were fed to rats daily. Once these chows were completely consumed, normal chows were given.

Pain Score Measurement

The measurement of pain score was performed according to Hayashida *et al.* (26). The number of pain-related responses, represented by vocalizations, was recorded during 10 flexions of the tarsotibial joints of the adjuvant-injected paw. Results were expressed as the mean number of vocalizations.

Splenocyte Preparation

At Day 14 and 28 after arthritis induction, splenocytes of each rat of group *P. canaliculus*, Naproxen, olive oil and control were prepared as described previously (27). Briefly, spleens were removed aseptically and minced into tiny pieces. Single cell suspensions were prepared by gentle grinding of spleen pieces in RPMI 1640 medium

(Life Technologies, US). Splenocytes (mostly B- and T-cells) from the crude spleen cell suspension were recovered by using Ficoll-Hypaque® Plus lymphocyte isolation kit (Pharmacia Biotech, USA) as described in the manufacturer's manual. Recovered splenocytes (1.5 ml) were transferred into a sterile centrifuge tube and four volumes of pre-chilled 0.83% ammonium chloride (NH_4Cl) was added and incubated for 10 min for lysis of residual erythrocytes (28). Splenocytes were recovered by centrifugation at 3000 rpm for 5 min, washed with 8 ml pre-warmed (37°C) sterile PBS buffer (137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_47H_2O$, 1.4 mM KH_2PO_4, pH 7.4) and finally resuspended in appropriate volume of complete RPMI 1640 medium (10% fetal bovine serum, 100 units ml^{-1} of penicillin and 100 $\mu g\,ml^{-1}$ of streptomycin, supplemented with 0.3 mg/ml L-glutamine and 25 mM HEPES).

Cell Count and Viability Staining

Resuspended splenocytes were stained with 0.4% Trypan blue exclusion dye (0.4 g Trypan blue in 100 ml PBS buffer) at ratio 1: 1. Number of splenocytes was counted and calculated with the aid of a hemocytometer. More than 99% of splenocytes were viable. Except stated otherwise, splenocytes were diluted to a working population of 5×10^6 cells ml^{-1} in all experiments.

ELISA Assay for Cytokines

A total of 5×10^6 splenocytes ml^{-1} were seeded in 24-well plate; a suboptimal concentration (1.25 $\mu g\,ml^{-1}$) of lipopolysaccharide (LPS) (Sigma, St Louis, MO, USA) was used to prime the splenocyte culture. Splenocytes were incubated in 37°C incubator with 80% humidity and 5% CO_2 atmosphere condition. Supernatant was collected at 10 and 24 h after LPS activation and stored at −80°C until later measurement. Interleukin-10 (IL-10), tumor necrosis factor-alpha (TNF-α) and interferon-gamma (IFN-γ) were measured by enzyme-liked immunosorbent assay (ELISA) (BioSource, CA, USA) as described in the user's manual.

Statistical Analysis

Experimental data were compared to the ones of the control group and analyzed by Student's *t*-test with one way ANOVA.

Results

Photographic and Radiographic Analysis of AIA Rat Paws

Figure 1 shows photographs of the right hind paw of rat at different times after AIA induction. Figure 1A was taken at 14 days after AIA induction. Swelling of the whole right hind paw, especially around the tarsotibial joints, is significant as shown in the photo. X-ray photography of the same paw at Day 21 after AIA reveals severe periarticular soft tissue swelling, joint subluxation and periostal new bone formation (Fig. 1B). The degree of arthritis between control group and Lyprinol® group shows no difference during the whole 28-day course of the experiment. However, 1 year after the induction of AIA, deformation of joints and lesions are still observable in the olive oil-control group (Fig. 1C). Conversely, deformation of joints and lesions

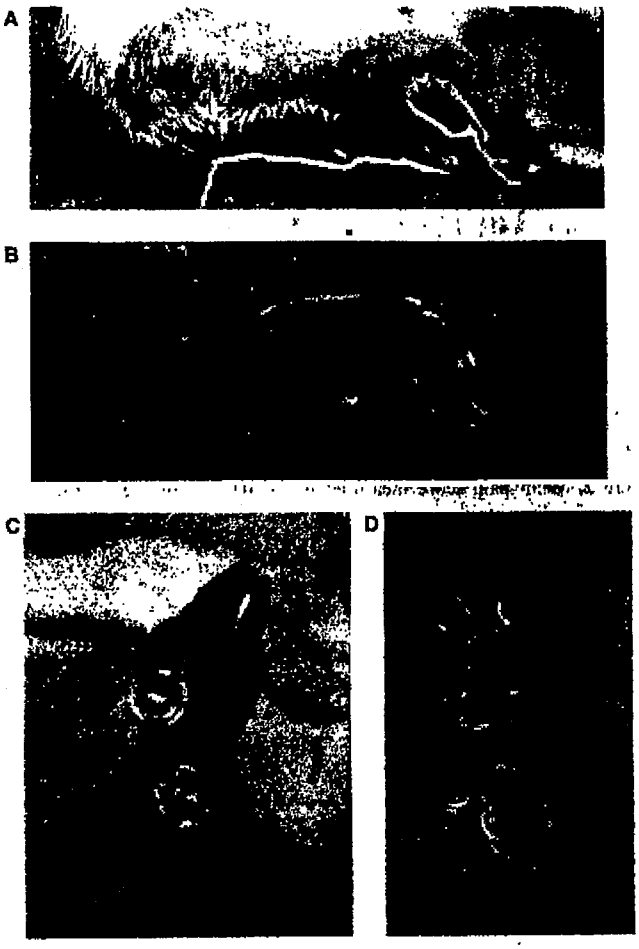

Figure 1. Photographic and radiographic analysis of adjuvant-induced arthritis (AIA) rats. (A) Photograph of the right hind paw taken 14 days after AIA induction. Swelling and inflammation of the paws were observed, especially around the ankle region. (B) Radiographic analysis of the same right hind paw at Day 21 after AIA induction. Note the swelling of soft tissues as well as bone deformation around the ankle region. (C) Photograph of the right hind paw in one of the representative rats in the olive oil group at 1 year after AIA induction. Toes and footpad are deformed, with multiple lesions. (D) However, rats fed with Lyprinol®-for 1 year recovered much better. Photograph of the right hind paw in one of the representative rats in the Lyprinol® treatment group showed only mild joint deformation and no lesion.

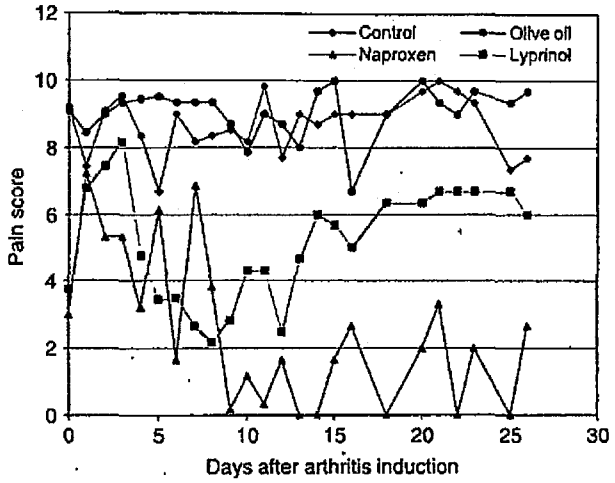

Figure 2. Mean pain score measured by 10 flexions of the tarsotibial joints of adjuvant-injected paw ($n = 6$), as described in Methods. Note that Lyprinol® effectively controlled pain between Day 4 and Day 12 when compared to control and olive oil groups.

are not visible in the Lyprinol®-long-term treatment group (Fig. 1D).

Pain Score Measurement

Pain score measurement is a widely used and reliable method to reflect the effectiveness of different treatments on AIA (26). It is one of the commonly used methodologies to measure level of pain (29,30,31). As shown in Fig. 2, the NSAID Naproxen effectively maintained the pain score of AIA rats at a relatively low level during the whole course of the experiment. Compared to the ones of the control and olive oil-treated groups, Lyprinol® effectively lowered the pain score of AIA rats from Day 4 to Day 26 after induction of arthritis. The effect of Lyprinol® was most pronounced between Day 4 and Day 12, with an effect comparable to that of Naproxen. The effect of Lyprinol® began to wane after Day 14, but still maintained the pain score at a significantly lowered level when compared to the control and olive oil-treated groups. Our results clearly demonstrated that Lyprinol® is very useful in controlling pain of severe arthritis, especially during the early and intermediate phase of AIA.

Level of Pro-inflammatory Cytokines Tumor Necrosis Factor-alpha and interferon-gamma

At Day 14 after induction of arthritis, splenocytes from Lyprinol®-fed rats were harvested and pro-inflammation cytokines levels were determined. As shown in Fig. 3, TNF-α level of Lyprinol® group was greatly decreased from 3.07 ± 0.168 to $1.71 \pm 0.405\,\text{ng ml}^{-1}$. Besides, production of another pro-inflammatory cytokine, IFN-γ, was significantly decreased. As shown in Fig. 4,

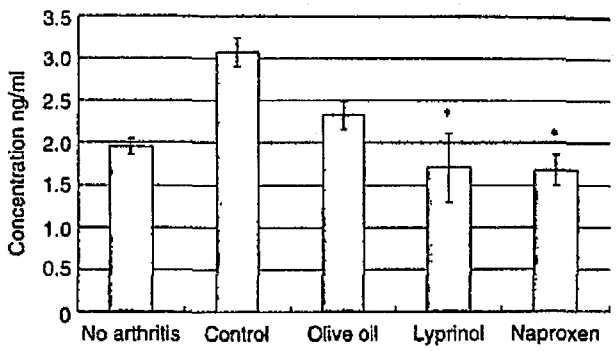

Figure 3. Amount of tumor necrosis factor-alpha (TNF-α) produced by LPS-stimulated splenocytes 14 days after arthritis induction. Lyprinol® controlled the amount of TNF-α production to a level similar to the one found in rats with no arthritis, and was as effective as the NSAID Naproxen. Values shown here are mean ± SEM ($n = 6$). $^*P < 0.05$.

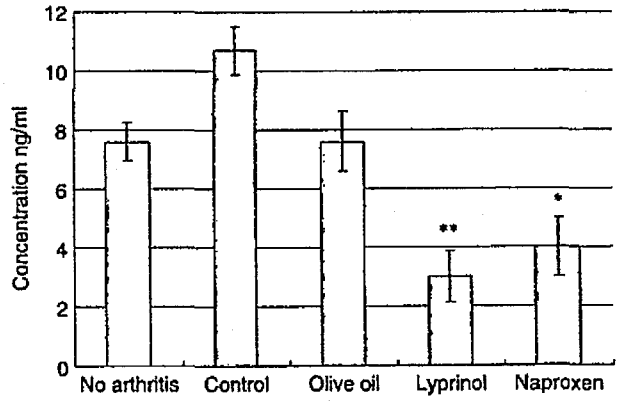

Figure 4. Amount of interferon-gamma (IFN-γ) produced by LPS-stimulated splenocytes 14 days after arthritis induction. Lyprinol® effectively reduced the amount of IFN-γ produced, when compared to control and olive oil groups. Strikingly, Lyprinol® was even more potent than the NSAID Naproxen in controlling the IFN-γ level. Values shown are mean ± SEM ($n = 6$). $^*P < 0.05$; $^{**}P < 0.001$.

the IFN-γ level of the Lyprinol® group, when compared to control, was dramatically decreased from level 10.7 ± 0.8 to $3.0 \pm 0.9\,\text{ng ml}^{-1}$.

Level of Anti-inflammatory Cytokine Interleukin-10

Figure 5 shows that the AI cytokine IL-10 increased at Day 28 after AIA. Although there is no statistically significant difference between the levels observed in the control group and the Lyprinol® group, the increase of IL-10 in the Lyprinol® group almost reached levels observed in the positive control group (Naproxen).

Discussion

Pain score measurement is a widely used and reliable method to reflect the effectiveness of different treatments

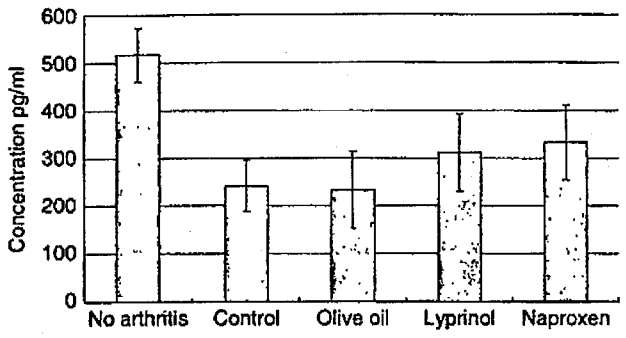

Figure 5. Amount of interleukin-10 (IL-10) produced by LPS-stimulated splenocytes after 28 days of arthritis induction. Lyprinol® can increase the amount of IL-10 production, an anti-inflammatory cytokine, although the difference was not statistically significant. Values shown are mean ± SEM ($n = 6$).

on AIA (26). It is one of the commonly used methodologies to measure level of pain. AIA in the rat is a standard model accepted for the study of inflammation and its control by medications or supplements (32). We used this model in order to get a better understanding of mechanisms resulting in AI effects of the lipid extract of *P. canaliculus*, the green-lipped mussel from New Zealand (Lyprinol®). A number of studies have been reported and/or published on this product in terms of its composition, its effects when compared with other drugs or lipids of diverse origins, its efficacy in several animal models and its effects on symptoms of OA and asthma.

Since the major complaint of patients suffering from acute exacerbations of chronic inflammatory disease is pain, we evaluated the analgesic effect of the lipid extract in the AIA rat model. We also measured levels of pro-inflammatory and anti-inflammatory cytokines. We compared results observed of Lyprinol, with a sham group (no drugs), an olive oil group (negative control), a non-steroid AI drug (NSAID) and a Naproxen (positive control) group. We also observed a group of rats as reference that were not treated with adjuvant.

Recent studies on pain, both in humans (33–36) and animals (34,37,38) have demonstrated the active role of IL-6 in inducing pain, as well as IL-1β, IL-8, TNF-α (TNF-R1 receptor) and IFN-γ. Conversely, IL-10 is associated with control of pain.

Lyprinol® exhibits a significant effect controlling pain in several recent clinical studies. The group at the University of Hong Kong (8) conducted the first randomized controlled trial to evaluate the effects of Lyprinol®, on signs and symptoms and quality of life (QOL) of patients with knee OA. Their results showed that Lyprinol® over 6 months in patients with knee OA is perfectly tolerated and associated with a decrease in pain perception after 2 months when compared to placebo. The visual analog scale (VAS) for pain score was significantly reduced from 63.0 at baseline to 55.5 at Week 4 ($P = 0.046$), 51.2 at Week 8' ($P = 0.003$) and 49.7 at Week 12 ($P = 0.001$). There was a greater significant reduction in VAS pain score following adjustment for change in amounts of paracetamol (acetaminophen) used in patients who received the lipid extract when compared with controls at Week 8 ($P = 0.035$), Week 12 ($P = 0.032$) and Week 24 ($P = 0.045$).

Another multi-center clinical study in Korea on patients with OA of the hip and knee found that the average VAS score, Lequesne functional index, global assessment by patients and global assessment by physician were all significantly improved (7). After a 4- and 8-week treatment period, 53–80% of patients experienced significant pain relief, and improvement of joint function. There was no reported adverse effect during this clinical trial (7).

A third study was conducted in Germany on 50 adults with inflammatory rheumatoid arthritis (21). The number of painful joints was reduced from 4.18 ± 2.80 at baseline by 0.30 ± 0.81 points (7.2%) after 6 weeks and 0.60 ± 1.18 points (14.4%) after 12 weeks ($P = 0.012$ and $P = 0.001$). For a sub-parameter 'number of painful small joints', a highly significant reduction of 9.3% after 6 weeks and 18.6% after 12 weeks was observed ($P = 0.022$ and $P = 0.002$). Analgesic medications were reduced or stopped in 21 subjects who required these at the beginning of the trial.

Our study of the mean pain score measurement by ten flexions of the tarsotibial joints of adjuvant-injected paw ($n = 6$) did demonstrate a significant reduction of pain in AIA rats treated with Lyprinol®, or Naproxen. However, this reduction differed according to the product used to treat the rats: pain scores in Naproxen and Lyprinol® groups initially increased; but they decreased significantly and gradually in the Lyprinol® group to reach a nadir (score = 2) at Day 8, while rats in the Naproxen group experienced a more erratic pain reduction with a significant nadir (score = 0) at Day 9. However, the pain score started differing significantly after Day 9: the Lyprinol® group experienced a progressive increase in pain scores, reaching its initial level at Day 15; while the Naproxen group remained essentially pain-free during the rest (total days = 26) of the experiment. This curve, transposed to the human time scale, is quasi-identical to the pain scale observed by Lau *et al.* (8) where the maximum reduction in pain was observed after approximately 8–12 weeks of treatment in patients with OA. It may well be due to an 'escape' mechanism from the production of pro-inflammatory cytokines that tend to increase despite treatment with Lyprinol®; it may also be due to other inflammatory mechanisms of AIA (and/or OA).

The cause of pain severity was confirmed by aspects of the inflamed paw when compared with the non-injected one, and comparative radiological images. X-rays confirmed the presence of major osteo-cartilaginous destruction as well as major swelling of the affected paw.

Pro-inflammatory cytokine production (TNF-α, IFN-γ) was significantly controlled by Lyprinol® (Day 14), as well if not better than Naproxen. IL-10, an 'anti-inflammatory' cytokine was increased in the Lyprinol® and in the Naproxen group, but did not reach statistical significance. Extra-virgin olive oil was ineffective in our study. More importantly, after 1 year, rats that were treated with Lyprinol® had a normal AIA paw, while untreated rats had severe inflammatory, ulcerative lesions.

Conclusion

The lipid extract of *P. canaliculus* is known as a powerful AI product in animal models and human diseases (asthma, arthritis). We have confirmed in the adjuvant-induced arthritis rat model, that this product (Lyprinol®) is an analgesic, as active as Naproxen during the early phase of treatment. AIA is a loco-regional highly inflammatory experimental condition, with systemic repercussions. Lyprinol® also controls as well as Naproxen the levels of pro-inflammatory cytokines (TNF-α, IFN-γ) in AIA rats; it increases the production of the AI cytokine IL-10 in AIA rats. Lyprinol®, was perfectly tolerated, and AIA rats treated with Lyprinol® were lesion-free after 1 year, while untreated controls had major inflammatory, ulcerative lesions. The lipid extract of *P. canaliculus* seems to be uniquely safe and effective in the treatment of pain, both spontaneous (due to inflammation) and triggered by torsion of the affected joint, in AIA in rats.

Acknowledgements

Prof. Georges M. Halpern (MD, PhD) initiated this study; organized it and helped to shape the manuscript. He should have been the leading author. We thank Pharmalink International Company Ltd, Burleigh Heads, QLD, Australia, for supplying the lipid extract of *P. canaliculus* (Lyprinol®) and the placebo, and for an unrestricted educational and research grant that supported our study in part.

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Efficacy and tolerability of mussel-Lyprinol® omega-3-complex on inflammatory rheumatoid disorders

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ABSTRACT

This 12 week drug monitoring study evaluated the effects of Sanhelios mussel-Lyprinol® omega-3-complex, on 50 adult men and women suffering from inflammatory rheumatoid arthritis. 34 of the 50 patients required medicinal treatment before and during the study. Upon completion of the study, for 21 of the 34 subjects (64 %) current drug therapy could be reduced or terminated. 13 of those did not even require further therapy. At the end of the treatment period, 38 % of all subjects were regarded as being free from disorders and the number of subjects suffering from severe pain was significantly decreased from 60% (at baseline) to 25 % (at completion of the trial). A significant positive effect was observed for all investigated parameters. Sanhelios mussel-Lyprinol® omega-3-complex was generally very well tolerated, with only one, non-serious adverse event (mild nausea) observed, which can probably be related to the study medication. Sanhelios mussel-Lyprinol® omega-3-complex , therefore, proved to be an effective and very well tolerated dietary supplement for the treatment of inflammatory rheumatoid arthritis.

Keywords: Lyprinol®; omega-3-complex, Vitamin E; inflammatory, rheumatoid arthritis; dietary supplement

INTRODUCTION

Rheumatism is comprised of a group of mostly chronic diseases, such as degenerative joint disorders (osteoarthritis), rheumatoid arthritis, weak tissue rheumatism (Fibromyalgia) and osteoporosis along with a drawing pain. In practice, therapeutic measures are usually combined. Apart from drug therapy, physical and operative treatment, nutrition is playing a crucial, as well as more and more recognized and accepted role, in therapy and prevention [5]. In particular, omega-3 fatty acids Eicosapentaen- (EPA) and Decosahexaen acids DHA), occurring in fish and mussels in large quantities, have been proven to reduce inflammatory and degeneration processes [2, 6, 7]. Persons with inflammatory rheumatoid disorders are observed to have an

increased demand for omega-3 fatty acids. With normal food intake (high proportion of meat, low proportion of fish) too much omega-6 fatty acids and too little fish are ingested. Vitamin E, biologically active tocopherols, were also shown to have a positive effect on the prevention of rheumatoid disorders [1]. Furthermore, Lyprinol® (a lipid extract of Perna canaliculus), that has proved to reduce joint pain and joint swelling as well as morning stiffness in patients suffering from inflammatory rheumatoid disorders [4, 3]

This clinical trial investigated the effects and tolerance of Sanhelios mussel-Lyprinol® omega-3-complex, a combination of omega-3-complex, Lyprinol®, and vitamin E on 50 subjects with inflammatory rheumatoid disorders.

METHODS

This study included 50 adults (25 men, 25 women), from 29 to 73 years old (mean: 59.2), with joint disorders, who met the following inclusion criteria: Rheumatoid joint disorders, a minimum level of moderate pain and morning stiffness. The exclusion criteria were pregnancy, breast-feeding or severe symptoms of rheumatism requiring immediate treatment. The diagnoses were confirmed in an examination by a physician. The history of the disorders varied from 0.67 to 21 years (mean: 8.1 ± 6.0 years). 30 subjects showed a recurrent course of rheumatoid disorder, with a mean period of time of the last recurrent of 1.4 ± 1.8 years. 34 subjects were treated with medication before and during the study period. The study complied with the recommendations of the German Association for Phytotherapy (Gesellschaft für Phytotherapie) regarding the performance of drug monitoring studies.

For the first two days of the individual trial period of 12 weeks, participants took one capsule each of Sanhelios mussel-Lyprinol® omega-3-complex in the morning and in the evening. From day three, the dosage was increased to 2 times 2 capsules per day for the duration of the study period. One capsule of Sanhelios mussel-Lyprinol® omega-3-complex contains 458 mg fish-oil concentrate (50% EPA, 50% DHA), 35 mg Lyprinol® (a lipid extract of Perna canaliculus)and 5 mg Vitamin E. Sanhelios mussel-Lyprinol® omega-3-complex is a dietary food supplement supporting the treatment of inflammatory rheumatoid arthritis. Assessments took place on day one, when baseline values were determined, after 6 weeks and at the completion of the trial after 12 weeks, or upon premature termination of the trial. The resulting parameters were changes in morning stiffness, joint pain, and the degree of pain intensity at examinations two and three compared to the baseline values.

RESULTS

Evaluation of the results is based on an intention to treat analysis. Of the 50 subjects initially enrolled, one did not complete the treatment period and left at day 70. All other subjects completed the trial.

Efficacy

The duration of morning stiffness was reduced from 13.7 ± 5.9 minutes at baseline by 1.3 ± 5.2 minutes (9.5 %) after 6 weeks and by 2.1 ± 3.8 minutes (15.3 %) at the completion of the trial. The reduction of morning stiffness after 6 weeks and after 12 weeks is highly significant ($p = 0.005$ and $p \leq 10^{-3}$). Similar results could be obtained for the outcome criteria "the number of painful joints" and "the number of swollen joints". The number of painful joints was reduced from 4.18 ± 2.80 at baseline by 0.30 ± 081 points (7.2 %) after 6 weeks and 0.60 ± 1.18 points (14.4 %) after 12 weeks (Fig. 1). Both values were statistically significant ($p = 0.012$; $p = 0.001$). The positive effect of mussel-Lyprinol® omega-3-concentrate was even more pronounced in the reduction of the "number of painful small joints", than for the "number of painful large joints". For the sub-parameter "number of painful small joints", a highly significant reduction ($p = 0.022$; $p = 0.002$) of 9.3 % after 6 weeks and 18.6 % after 12 could be observed (Fig. 1).

The subjects and the physician independently evaluated the pain intensity according to a 5 point scale (0 = no pain to 4 = very severe pain). During the course of the trial, a successive reduction of pain was observed. The reduction of pain was highly significant after the second assessment at 6 weeks. According to the physician's evaluation, in 44 % of the subjects ($n = 22$) the pain intensity was alleviated from moderate or severe pain intensity to mild pain intensity by the completion of the study (Fig. 2). According to the subjects self-rating this was achieved in 38 % ($n = 19$) of all subjects. The pain reducing effect of Sanhelios mussel-Lyprinol® omega-3-complex was even more pronounced in subjects who suffered from severe pain at the beginning of the trial. There the number of subjects was reduced from 29 to only 12 (evaluation of the physician) and from 31 to 13 (evaluation of the subjects), respectively (Fig. 2). For most of these subjects the improvement in pain intensity was observed at the second assessment after 6 weeks. Drug treatment could be reduced or even terminated for 21 of the 34 subjects who required medicinal treatment at beginning of the trial. On completion of the study, none of the subjects were completely free from pain. However, 19 subjects (38 %) were diagnosed being free of disorders and not requiring further treatment. In the global evaluation of efficacy, 57.1 % of the subjects were evaluated as "very good" or "good".

Tolerability

During the 12 week trial, the subjects reported a total of seven adverse events. None of these were severe. In one case, where the subject reported mild nausea at the beginning of the trial, the adverse event can be probably attributed to the study medication. The effect might be due to an aversion to fish oil. However, this improved during the course of the trial.

Global evaluation of tolerability was equally assessed as "very good" or "good by 98 % of the subjects and the physician.

DISCUSSION

Rheumatoid arthritis, with a prevalence of 0.5 to 1 % of the population, is the most common of systemic inflammatory rheumatic disorder, and predominantly affects the musculoskeletal system. Parthenogenesis is mostly unknown, therefore, therapy is primary focused on unspecific suppression of the inflammatory processes. Apart from medicinal therapy, nutrition is recognized as playing a major role in prevention as well as treatment. Subjects enrolled in this trial, investigating the efficacy and tolerability of Sanhelios mussel-Lyprinol® omega-3-complex, suffered from rheumatoid arthritis (78 %) and rheumatoid arthrosis (22 %). The subjects have suffered from these disorders for an extended period (mean: 8.1 years) and 34 (64 %) of them required medicinal treatment prior to and during the course of the trial. One of most important results obtained from this trial is that for 21 of 34 subjects who required medicinal treatment, current medicinal therapy could be reduced by the end of the treatment period. 13 of these those 21 subjects did not even require further therapy. In particular, when regarding the adverse effects of generally prescribed medication, this can be regarded as a major success for treatment with the dietary food supplement Sanhelios mussel-Lyprinol® omega-3-complex. A great reduction of joint pain and swelling, as well as morning stiffness, was not observed and was not expected. However, the improvements of 5.8% to 26 %, observed in the investigated parameters, were all statistically significant and strongly argue for the positive effects of the treatment. Furthermore, it should be taken into account that 38 % of the subjects were regarded as being free from disorders at the end of the treatment period and that the number of subjects suffering from severe pain was reduced from 60% to 25 % (Fig. 2). In the global evaluation of efficacy, 57.1 % of the subjects were evaluated as "very good" or "good". Taking into account that the positive effects increased during the study period, this percentage may be even higher when Sanhelios mussel-Lyprinol® omega-3-complex is taken for a longer period of time. The study preparation was very well tolerated. 98 % of the subjects evaluated the safety of Sanhelios mussel-Lyprinol® omega-3-complex as "very good" or "good. Of the seven reported adverse events, only one (mild nausea) could be reasonably related to the study medication.

Conclusions: Sanhelios mussel-Lyprinol® omega-3-complex showed significant positive effects in the treatment of inflammatory rheumatoid disorders. These positive effects may be more pronounced when taken for a longer period than was investigated in this study. Sanhelios mussel-Lyprinol® omega-3-complex was also very well tolerated by the subjects. It, therefore, can be recommended as a dietary food supplement in the treatment of inflammatory rheumatoid disorders.

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Fig. 1. Changes in number of swollen and small hurting joints.

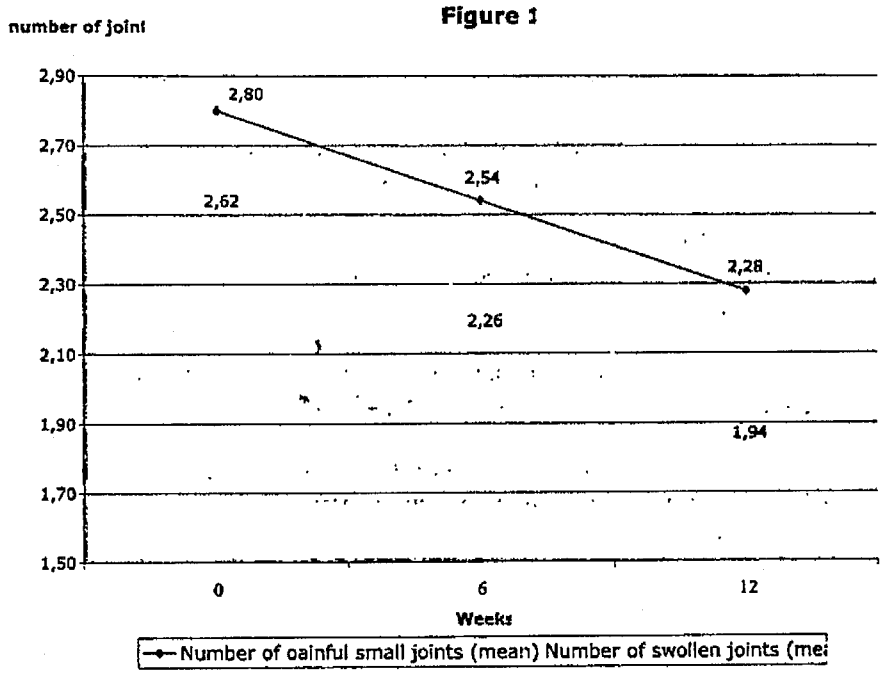
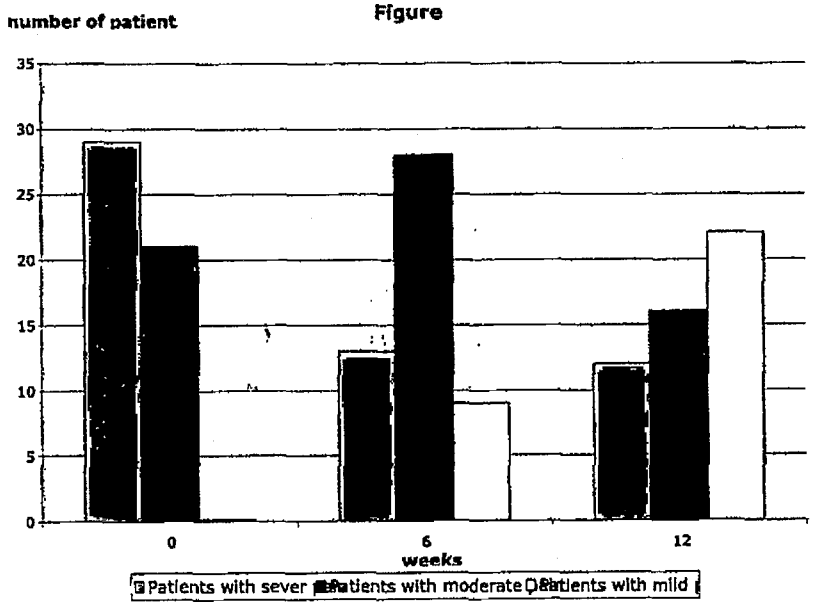


Fig. 2. Changes in pain intensity.



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Novel anti-inflammatory ω-3 PUFAs from the New Zealand green-lipped mussel, *Perna canaliculus*

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Abstract

The present study has identified in the marine mollusc, *Perna canaliculus*, an homologous series of novel omega 3 polyunsaturated fatty acids (ω-3 PUFA) with significant anti-inflammatory (AI) activity. The free fatty acid (FFA) class was isolated from a supercritical-CO_2 lipid extract of the tartaric acid-stabilised freeze-dried mussel powder by normal phase chromatography, followed by reversed-phase high performance liquid chromatography (RP–HPLC). The RP–HPLC involved separation based on carbon numbers, followed by argentation–HPLC (Ag–HPLC) of the methyl esters based on degree of unsaturation. Identification of the FFA components was performed using gas chromatography (GC) with flame ionisation detection, and individual structures were assigned by GC-mass spectroscopy (GC-MS). Inhibition of leukotriene production by stimulated human neutrophils was used as an *in vitro* screening method to test the AI activity of the purified PUFAs. A structurally related family of ω-3 PUFAs was identified in the most bioactive fractions, which included C18:4, C19:4, C20:4, and C21:5 PUFA. The C20:4 was the predominant PUFA in the extract, and was a structural isomer of arachidonic acid (AA). The novel compounds may be biologically significant as AI agents, as a result of their *in vitro* inhibition of lipoxygenase products of the AA pathway.

Keywords: Anti-inflammatory; Eicosanoid; Lipoxygenase; Lyprinol®; ω-3 PUFA; *Perna canaliculus*; Supercritical fluid extract

1. Introduction

Eicosanoids of the cyclooxygenase (COX) and lipoxygenase (LO) pathways of arachidonic acid (AA) metabolism are important modulators of inflammation (Bogatcheva et al., 2005). Often, excessive inflammatory responses progress to pathogenic states requiring pharmacological intervention. Elucidation of the AA pathways has led to understanding of modes of action of traditional anti-inflammatory (AI) drugs, and enhanced research into specific inhibitors of the AA pathways for further drug development (Morris et al., 2006). The reported side-effects and contra-indications of current AI drugs have lead to investigations into natural products for safer and more effective alternatives (Calder, 2006).

An area of recent investigation is the AI activity of *Perna canaliculus* (Bivalvia: Mytilidae), a marine mollusc commonly known as the green-lipped mussel of New Zealand. Lyprinol®, a commercially available preparation of *P. canaliculus*, is the mussel oil obtained by carbon dioxide-supercritical fluid extraction (CO_2–SFE) (Macrides and Kalafatis, 2000) of the tartaric acid-stabilised mussel powder (Kosuge and Sugiyama, 1989), formulated with olive oil and vitamin E as an antioxidant. Clinical trials and *in vivo* rat assays have established the AI effectiveness of Lyprinol®. The first randomised trial of Lyprinol® in the treatment of arthritis in humans was reported by Gibson and Gibson (1998). They showed that 76% of patients with rheumatoid arthritis and 70% of patients with osteoarthritis benefited from Lyprinol®. It has been found that increased consumption of ω-3 PUFAs alter the generation of AA-derived inflammatory metabolites, and reduce inflammatory responses (Stamp et al., 2005). Lyprinol® has been shown to have greater AI activity than the more commonly used ω-3 PUFA-containing therapeutic oils (Whitehouse et al., 1997). When administered therapeutically or prophylactically to Wistar and Dark Agouti rats with antigen-induced polyarthritis or collagen (II)-induced autoallergic

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Table 1
Solvent program for the FFA group purification by preparative NP–HPLC

Segment	Time (min)	Hexane (%)	Tetrahydrofuran (%)
0	0	100	0
1	20	96	4
2	25	90	10
3	45	0	100
4	48.8	90	10
5	50	100	0

arthritis, Lyprinol® was a more effective AI agent (Effective Dose, $ED_{50} \leq 15$ mg/kg) than flaxseed, evening primrose, and fish oil ($ED_{50} \geq 1800$ mg/kg) (Whitehouse et al., 1997). Lyprinol® has been shown to inhibit leukotriene B_4 (LTB_4) production in calcium and ionophore-stimulated human neutrophils (Whitehouse et al., 1997), and in interleukin-4-induced human monocytes (Dugas, 2000). Inhibition of prostaglandin-E_2 production in activated human macrophages has also been observed (Whitehouse et al., 1997). It has been shown that the AI activity of the CO_2–SFE mussel oil resides predominantly in the FFA fraction of the oil, with the greatest activity being exhibited by the PUFA class (McPhee et al., 2001; McPhee et al., 2007). The identities of the active PUFAs are however unknown.

Isolation of active components from whole organisms requires several purification steps, with each step requiring a screening process to target the active components. Lipid extracts can be obtained by several methods, and SFE has been found to be effective in removing lipid fractions from solid or semi-solid material (Huang et al., 1994). Lipids obtained from whole organisms contain a large number of different lipid classes, each of which is itself heterogeneous. Therefore a sequential purification process is needed to purify and identify bioactive compounds from the lipid extracts. Gas chromatography offers a sensitive identification system for any novel or unusual compounds in the fractions. A sensitive and reliable assay of AI activity is the 5-LO inhibition assay (Cleland et al., 1990), which monitors the production by stimulated human neutrophils of pro-inflammatory leukotriene and hydroxy acid metabolites of the 5-LO pathway of AA metabolism.

In the present study, a simple purification procedure based on normal and reversed phase chromatography was carried out to isolate potential bioactive lipids from the FFA class of the CO_2–SFE oil from *P. canaliculus*. Novel fatty acids were detected by GC and structurally identified by GC-MS. Bioactivity was confirmed in an *in vitro* lipoxygenase-inhibition assay system.

2. Materials and methods

2.1. Chemicals

Solvents for all chromatographic procedures were of analytical grade quality and obtained from E. Merck (Darmstadt, Germany). Silica gel for column chromatography (Kieselgel 60, 230–400 mesh) and thin layer chromatography (TLC) plates (Kieselgel 60F254 nano DC) were sourced from Merck (Darmstadt, Germany). Lipid standards for thin layer chromatography (TLC) and HPLC analysis were obtained from Nu-Chek-Prep Inc., (Elysian, MO, USA).

2.2. Mussel extract

Tartaric acid stabilised green-lipped mussel (*P. canaliculus*) powder (McFarlane Marketing (Aust) Pty Ltd, Melbourne, Australia) was extracted for lipids by the procedure of SFE, utilising CO_2 as the extracting medium (Macrides and Kalafatis, 2000). Essentially, mussel powder (300 g) was charged to the pilot scale Super Critical Fluid Extraction Unit (Distillers MG Ltd. England, UK). Supercritical-CO_2 was delivered at a flow rate of 3.0 kg/h for two hours. The extractor temperature was set at 70 °C and the extractor pressure at 345 bar. The evaporator parameters were set at 40 °C and 35 bar. The extract presented as a concentrated oil, and was stored under nitrogen at −8 °C in amber vials to minimise autoxidation.

2.3. Open column flash chromatography

Initial fractionation to remove the highly polar mussel lipids was performed by open column flash chromatography using silica gel (Still et al., 1978). The mussel extract (4.5 g) dissolved in dichloromethane (DCM, 6.0 mL) was applied to 100 g of silica gel (230–400 mesh) in an open column (100×8 cm ID) fitted with a 1 L solvent reservoir (flow rate 175 mL/min). Separation of the lipid classes was achieved using a polarity gradient utilising two bed volumes of the following solvents; 100% DCM, 100% hexane, 10%, 50%, methyl-*tert*-butyl ether (MTBE) in hexane, 100% MTBE, and finally 100% methanol (MeOH) to remove the highly polar material. A total amount of 90 g of mussel oil was processed.

2.4. Silica gel NP–HPLC purification of FFA class of mussel extract

Group purification of the FFA class of the phospholipid-free mussel extract was obtained by silica gel preparative HPLC using normal phase (NP) chromatography. The phospholipid-free fractions collected from the low resolution column procedure were pooled, filtered through a 0.45 μm polyvinylidene hydrofluoride filter (Activon Scientific Products, Thornleigh, Australia) and evaporated to dryness. The sample (450 mg) re-dissolved in hexane diluent (900 μL) was applied to a Prep Nova-Pak® HR 60Å, 6 μm silica, 100 mm×40 mm (ID) column (Waters Chromatography Division Milford, MA, USA). Preparative HPLC analysis was performed using a Waters Delta Prep 3000

Table 2
Solvent program for the separation of FFA class by semi-preparative RP–HPLC

Segment	Time (min)	Water (%)	Acetonitrile (%)	Methanol (%)
0	0	13	87	0
1	35	13	87	0
2	40	0	100	0
3	42	0	0	100
4	57	0	50	50
5	59	13	87	0

Table 3
Gradient program for the separation of FAME by semi-preparative Ag–HPLC

Segment	Time (min)	A (%)	B (%)
0	0	70	30
1	31	8	92
2	35	8	92
3	43	0	100
4	53	0	100
5	55	70	30

A=DCM–DCE, 1:1 (v/v).
B=DCM–DCE–AcCN–MeOH, 40:40:10:10: (v/v/v/v).

solvent delivery system (flow rate 25 mL/min) with a 600E system controller (Waters Chromatography Division). The compounds were detected by an ACS Model 750/14 light scattering detector (Applied Chromatography Systems, Macclesfield, UK). The light scattering detector was set at an evaporator value (ESV) of +80 °C and a nitrogen delivery pressure of 20 psi. The data was collected on a PE Nelson Model 1020 Personal Integrator (The Perkin Elmer Corporation, Norwalk, CT, USA). Sample collection was achieved by inserting a stream splitter between the column outlet and detector inlet, with 5% of the effluent going to the detector. The solvent system (Table 1) was developed by using commercial standards of tristearin (Nutritional Biochemicals Corporation, Cleveland, OH, USA), cholesteryl palmitate, stearic acid, and cholesterol (Sigma). The retention times of these standards were used to identify the lipid classes of the mussel extract applied. The identity of each peak was also confirmed by TLC analysis.

2.5. Separation of FFA by reversed phase semi-preparative HPLC

The FFA material obtained from the NP–HPLC procedure was evaporated to dryness and re-dissolved in acetonitrile (AcCN)-tetrahydrofuran (THF) (1:2 v/v) before being passed through a nylon 0.45 μm filter (Activon). The multi-solvent system (Table 2) developed for the FFA separation utilised AcCN as the polar carrying solvent with water and MeOH introduced to alter the solvent strength and selectivity of the mobile phase. The RP–HPLC utilised an Ultrasphere™ C18, 80 Å, 5 μm, 25 cm × 10 mm (ID) column obtained from Beckmann (Palo Alta, CA, USA) with a C18 pre-column, 1 cm × 4.3 mm (ID) (Activon). Semi-preparative HPLC analysis was performed on an Applied Biosystems Chromatograph, Model 150A with a 1400 A ternary high pressure pump system (flow rate 1.5 mL/min) equipped with a model 1783 A detector controller unit (Applied Biosystems, Foster City, CA). The light scattering detector was set at an ESV of +45 °C and a gas delivery pressure of 14 psi with the data collected and stored on a 1020 Personal Integrator (Perkin Elmer). The sample was delivered through an automated sampling unit, WISP 712 (Waters Chromatography). Sample collection was achieved using a Gilson Model 202 programmable collector (Gilson Medical Electronics Inc., Middleton, WI, USA). The samples were collected in a preset time windows mode of collection with the column effluent diverted directly to the fraction collector.

2.6. Lipoxygenase inhibition assay

The fractions obtained from the RP–HPLC were evaporated to dryness and a known weight of each fraction was dissolved in 1 mL of MeOH. The *in vitro* assay was performed according to the method of Cleland et al. (1990). Briefly, the assay involved stimulation of human neutrophils with AA and calcium ionophore A23187, resulting in the production of the 5-LO

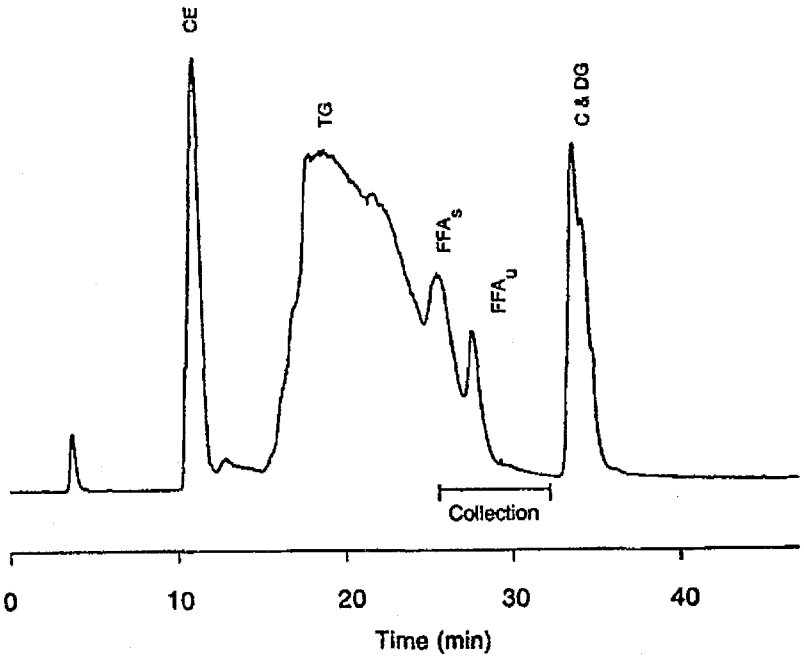

Fig. 1. Preparative NP–HPLC chromatogram of 450 mg of phospholipid-free mussel lipid extract. Chromatographic conditions: Nova Pak® silica preparative column, using the multi-solvent system shown in Table 1, and a flow rate of 25 mL/min. Light scattering detector settings: ESV +80 °C gas pressure 20 psi. The peaks identified were: CE, cholesterol esters; TG, triglycerides; FFA$_s$, saturated free fatty acid region; FFA$_u$, unsaturated free fatty acid region; C, cholesterol; and DG, diglycerides.

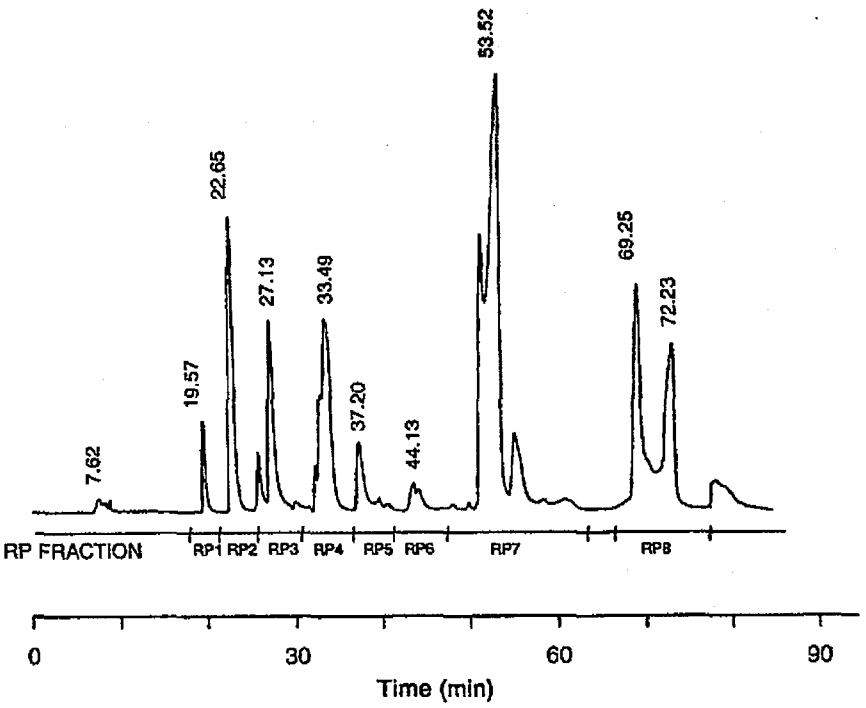

Fig. 2. Semi-preparative RP–HPLC chromatogram of mussel FFA class. Chromatographic conditions: Beckman™ C18 semi-preparative column, using the solvent system described in Table 2 and flow rate of 1.5 mL/min with 1 mg being applied. Light scattering detector settings: ESV, +45 °C gas pressure 14 psi. The retention times of the eluted FFA and the collection time windows are indicated.

metabolites; LTB_4, the two non-enzymic isomers, 6-*trans* LTB_4 and 6-*trans*,12-*epi* LTB_4, and 5-hydroxyeicosatetraenoic acid (5-HETE), which were quantified by HPLC. Samples were typically assayed at three dilutions, i.e. 1:100, 1:1000, and 1:10,000, with an inhibition of the production of the LO metabolites indicating bioactivity. Percent inhibition of control production of LO metabolites was calculated by: 100 × (concentration of LO metabolite of test sample/concentration of LO metabolite of control).

2.7. Analysis of fractions by gas chromatography

The FFA fractions from the chromatography procedures were dissolved in hexane (1 mL) and a C17:0 methyl ester internal standard (IS) (Nu-Chek-Prep) was added (fraction:IS, 5:1 w/w). The fatty acids were converted to their methyl esters (FAME) by reaction with BF_3–MeOH, as described in the Association of Official Analytical Chemists method 963.22 (AOAC, 1995). Briefly, 10 mL of 12% BF_3 in MeOH was added to 500 mg of FFA and refluxed for 60 min at 70 °C. A 20 mL aliquot of heptane was added and the reaction mixture refluxed for a further 10 min. The reaction was terminated by the addition of 15 mL of saturated sodium chloride. The FAME were extracted using 2 volumes of 50 mL MTBE. The organic layer was washed with 20 mL volumes of water until the solution was acid free. The extract was dried over anhydrous Na_2SO_4 and analysed by TLC. The unreacted material was removed by a clean-up procedure using 2 g of fine silica in a 10 mL syringe, and the flow rate (4 mL/min) was achieved by applying compressed air. The FAME were separated by a stepwise elution gradient utilising 8 mL of each of the following solvents; 100% hexane, 2%, 10%, 50% MTBE in hexane, and finally 100% MTBE.

GC analysis was performed using a Shimadzu model GC-17A with a flame ionisation detector set at 260 °C and linked to a Shimadzu Chromatopac integrator (Shimadzu Corporation, Tokyo, Japan), which was equipped with a split/splitless injector. The FAME (1 μL injections) were separated using a fused silica capillary column (50 m × 0.22 mm (ID), film thickness 0.2 μm) coated with BPX70 (biscyanopropyl polysiphenenylene-siloxane, SGE, Ringwood, Victoria, Australia), with helium as the carrier gas. The oven temperature was held at 120 °C for 1 min, then increased to 170 °C (at a rate of 5 °C/min) and held for 4 min, then increased to 220 °C (at a rate of 10 °C/min) and held for 17 min. The injector temperature was set at 260 °C and the linear velocity of the helium gas was 20 cm/min. Compounds were identified by comparison of the retention time to known FAME standards (Nu-Chek-Prep) and were quantified by comparison to the C17 internal standard peak.

2.8. Analysis by thin layer chromatography

A commercial TLC standard lipid mixture, containing cholesterol ester (CE), triglyceride (TG), cholesterol (C), phospholipid (PL), diglyceride (DG) and free fatty acid (FFA), together with the crude mussel extract and semi-purified fractions were applied to a silica gel plate. The plates were developed in a mobile phase of hexane-diethyl ether-acetic acid (80:20:2 v/v/v) until the solvent reached approximately 2 cm from the top of the plate (Christie, 1982). Lipid compounds were visualised by spraying with 10% $CuSO_4$, 8% H_3PO_4 in water and heating at 110 °C for 20 min

Table 4
Percent inhibition of the production of LO metabolites by RP fractions

Code	Dilution	Inhibition (%)[1]			
		6-*trans* LTB_4	6-*trans*,12-*epi* LTB_4	LTB_4	5-HETE
RPFA-1	1:100	13±5.2	13±4.7	2±3.8	0±23.2
	1:1000	0±5.7	0±5.1	3±4.0	0±15.9
	1:10,000	8±4.2	10±4.1	4±3.0	4±13.8
RPFA-2	1:100	100±0.7	100±0.0	100±0.8	100±3.1
	1:1000	24±1.1	40±1.1	47±1.1	3±7.1
	1:10,000	12±2.8	12±2.6	20±2.7	0±10.8
RPFA-3	1:100	94±1.6	100±2.4	79±3.0	100±7.2
	1:1000	29±4.5	43±3.9	13±3.0	12±16.3
	1:10,000	12±4.7	26±3.9	8±3.0	7±12.2
RPFA-4	1:100	100±2.7	100±2.4	100±2.2	100±7.2
	1:1000	43±4.5	58±4.0	25±3.1	23±13.4
	1:10,000	20±4.3	29±3.8	13±3.3	6±14.2
RPFA-5	1:100	97±3.7	100±2.4	89±3.3	83±11.3
	1:1000	25±4.8	43±4.3	13±3.0	0±19.7
	1:10,000	19±3.5	36±3.6	6±3.1	0±13.7
RPFA-6	1:100	22±2.3	33±2.1	16±2.0	10±9.3
	1:1000	0±4.6	0±4.0	0±2.4	0±14.6
	1:10,000	0±4.7	0±4.2	0±2.4	0±11.7
RPFA-7	1:100	44±3.5	59±3.3	22±3.3	22±11.6
	1:1000	0±4.4	6±4.1	0±3.5	0±13.6
	1:10,000	8±4.1	19±3.6	6±2.7	5±10.1
RPFA-8	1:100	26±1.1	43±1.0	14±1.4	3±7.0
	1:1000	11±2.2	12±1.6	14±1.3	0±7.2
	1:10,000	0±3.6	0±2.7	8±3.6	0±7.6
FFA[2]	1:100	89±2.1	100±0.6	100±0.8	89±10.1
	1:1000	0±3.3	0±2.6	0±2.1	0±13.6
	1:10,000	0±2.2	0±1.8	0±1.4	0±11.5

[1]Control values were, typically (ng/10^6 cells, mean±s.d, n=4): 6-*trans* LTB_4, 28.7±2.7; 6-*trans*,12-*epi* LTB_4, 21±2.4; LTB_4, 20.2±2.2; 5-HETE, 147±7.2. The activity of the test samples is expressed as the % inhibition of control production of LO metabolites (mean±s.d, n=4). [2]Total free fatty acids isolated by open column NP flash chromatography.

(Bitman and Wood, 1982). The analysis of the FAME was carried out by TLC in a solvent system of 4% MTBE in 96% hexane, with a visualising standard of palmitic methyl ester.

2.9. Argentation–HPLC (Ag–HPLC) separation of the FAME

The RP–HPLC fractions which were identified as bioactive and novel were individually purified by Ag–HPLC. These fractions (as their methyl esters) were evaporated to dryness, dissolved in 1, 2-dichloroethane (DCE) (1:10 w/v) and passed through a 0.45 μm nylon filter (Activon). The optimal separation system was developed by applying a commercial standard mixture (Nu-Chek-Prep 84) containing C16:0, C17:0, C18:0, C18:1, C18:2, C18:3, C20:0, C20:4 and C22:6 methyl esters. A gradient, multi-solvent system (Table 3) was developed with DCM–DCE (1:1 v/v) as the equilibrating solvent and a stepwise increase of AcCN–MeOH (1:1 v/v) to alter the solvent strength and selectivity of the mobile phase. The Ag–HPLC utilised a Chromsphere™ 5 μm, 25 cm × 10 mm (ID) C18 silver impregnated column (Chrompack International, Middelburg, Netherlands) with a 75 mm × 4.6 mm Valco Polar bonded phase (Chrompack) pre-column. The same instrumentation as the RP–HPLC was used with the detector setting ESV at +70 °C and a gas delivery pressure of 14 psi. The samples were collected in a preset time windows mode of collection with the column effluent diverted directly to the fraction collector.

2.10. GC-MS analysis of active FAME fractions from Ag–HPLC separation

The analysis was carried out using a Hewlett-Packard 5890 GC (Palo Alta, CA, USA) fitted with an BPX5 capillary column (12 m × 0.22 mm (ID), film thickness 0.33 μm, (SGE) inlet pressure of 5 psi. The oven temperature was held at 75 °C for 2 min, and then heated at 30 °C/min to 300 °C, with a final holding time of 9 min. Splitless injections were used with an injector temperature of 250 °C.

The GC-MS was carried out in both electron impact (EI) and chemical ionisation (CI) modes with both data sets providing supportive data. The EI-MS was performed using the above GC conditions and utilising a quadruple Finnigan MAT INCOS-50 MS, with an interface temperature of 280 °C and the ion source temperature of 180 °C. The scan range was 50 to 500 Da at 0.6 s/scan. The positive ion CI-MS using methane as the ionisation gas was performed with the same conditions as described above on a Hewlett-Packard 5890 GC (Palo Alta, CA, USA) and a Finnigan MAT TSQ-70 MS detector.

3. Results

3.1. Supercritical fluid extraction

The maximum yield of CO_2–SFE extractable oil from *P. canaliculus* freeze dried mussel powder was 4.76% w/w. The mussel extract was orange-amber with a viscous waxy appearance at ambient temperature. Analysis of the oil by TLC afforded several lipid classes including cholesterol esters (CE), triglycerides (TG), free fatty acids (FFA), diglycerides (DG), cholesterol (C), phospholipids (PL) and monoglycerides (MG).

3.2. Group separation of the FFA class of the mussel extract

A representative chromatogram of the fractions obtained in the preparative NP–HPLC system is shown in Fig. 1, with the elution order of the lipid classes indicated (i.e. CE, TG, FFA, DG and C). The lipid classes were identified in the chromatogram by comparison of the relative retention times to commercial standards, as well as by TLC analysis.

The FFA class was eluted in a retention volume of 640–775 mL, and divided into two regions. The first region designated "FFA_s", which eluted close to the TG, was identified by GC as the saturated FFA component. The second region designated "FFA_u" had a longer retention time, and contained the unsaturated FFA component. The later region was determined to be the more biologically active fraction by the *in vitro* assays. The collection of the FFA fraction commenced from the middle of the first region in order to avoid excessive carry-over of the TG class. The overall yield of the collected FFA fraction was 10% of the total lipid material.

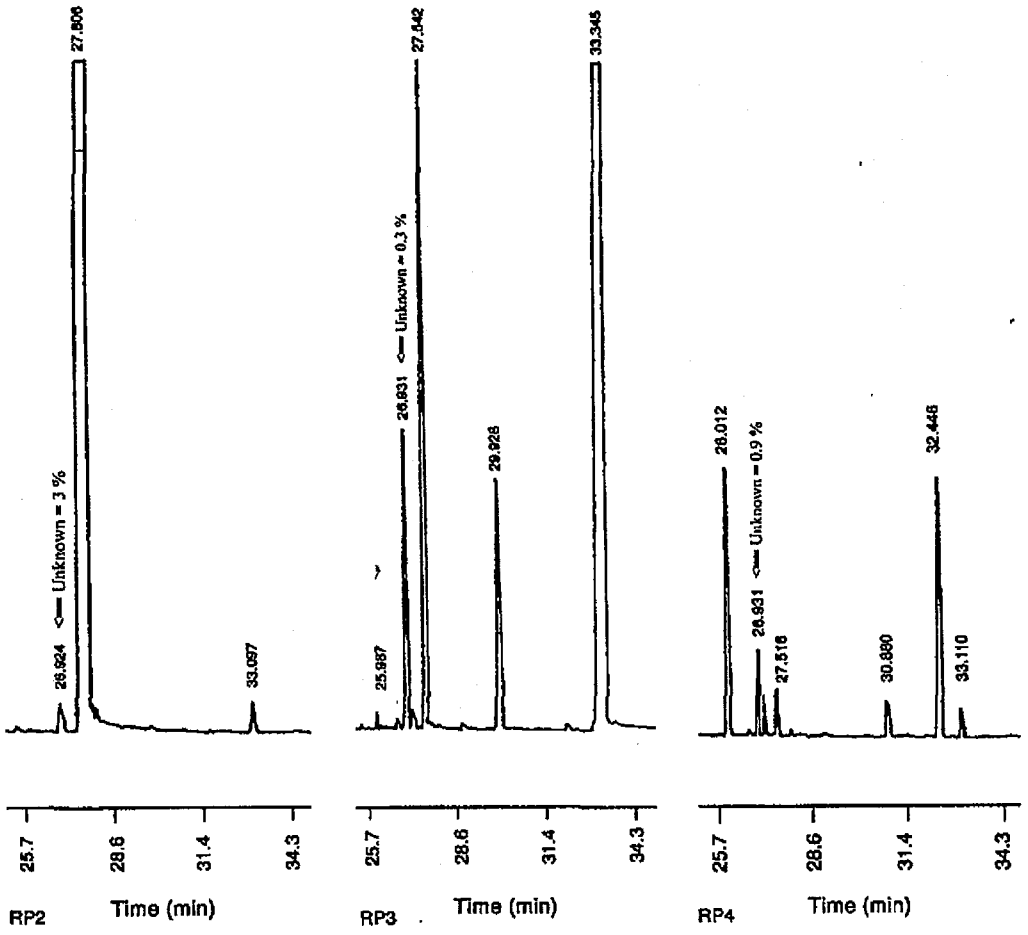

Fig. 3. The significant regions of GC chromatograms of the bioactive fractions RP2, RP3, and RP4, which correspond to time windows shown in Fig. 2. Chromatographic conditions: BPX70 capillary column (50 m × 0.22 mm (ID), 0.2 μm), injector temperature 260 °C, using the temperature program in methods Section 2.7. The common unknown compound is expressed as a percentage of the total FAME in each of the fractions.

3.3. Semi-preparative reversed-phase–HPLC

A preliminary separation of the FFA fraction obtained from NP-chromatography was carried out by RP–HPLC. Fig. 2 indicates the separation achieved by the RP–HPLC with the relative elution times shown above each peak. The fractions were collected according to the time windows shown in Fig. 2 and coded "RP1" through to "RP8", and screened for AI activity in the *in vitro* leukotriene inhibition assay.

3.4. Leukotriene in vitro assay of RP1 to RP8

The RP–HPLC fractions RP1-RP8 and the FFA class were screened by the *in vitro* assay and the results of this assay are summarised in Table 4. The FFA class contained activity at the 1:100 dilutions only. Fractions RP2–RP5 exhibited the most inhibition of the LO products formed (at all dilutions), thus indicating that bioactive compounds were located in these fractions.

3.5. Analysis of bioactive fractions by GC

Fractions RP2–RP5 were analysed by GC as their methyl esters. Fraction RP5 contained known PUFA but no novel compounds when compared to the retention times of commercial standards, therefore it was not further purified. The fractions RP2–RP4 all contained unusual compounds, and are shown in Fig. 3 which illustrates the significant region of each of the GC chromatograms. The chromatogram of the profiles of RP2, RP3, and RP4 shared a common unknown compound at the retention time of 26.93 min under the experimental conditions. This unusual FAME had separation characteristics which indicted it was 20 carbons in length with 4 unsaturated double bonds (C20:4). Fractions RP2, RP3 and RP4 were further purified by Ag–HPLC.

3.6. Semi-preparative Ag–HPLC separation of RP-FAME

The bioactive fractions were converted into their methyl esters, with a 93% conversion. Using the 12% BF_3 in MeOH method, RP2 (365.6 mg), RP3 (519.5 mg), and RP4 (757.2 mg) were individually converted to obtain 340.0 mg, 405.6 mg and 673.9 mg, respectively.

The Ag–HPLC silver impregnated C18 column was able to separate the FAME by their degree of unsaturation, with the most saturated compound being eluted first. The separation of the fractions RP2, RP3 and RP4 are indicated by a

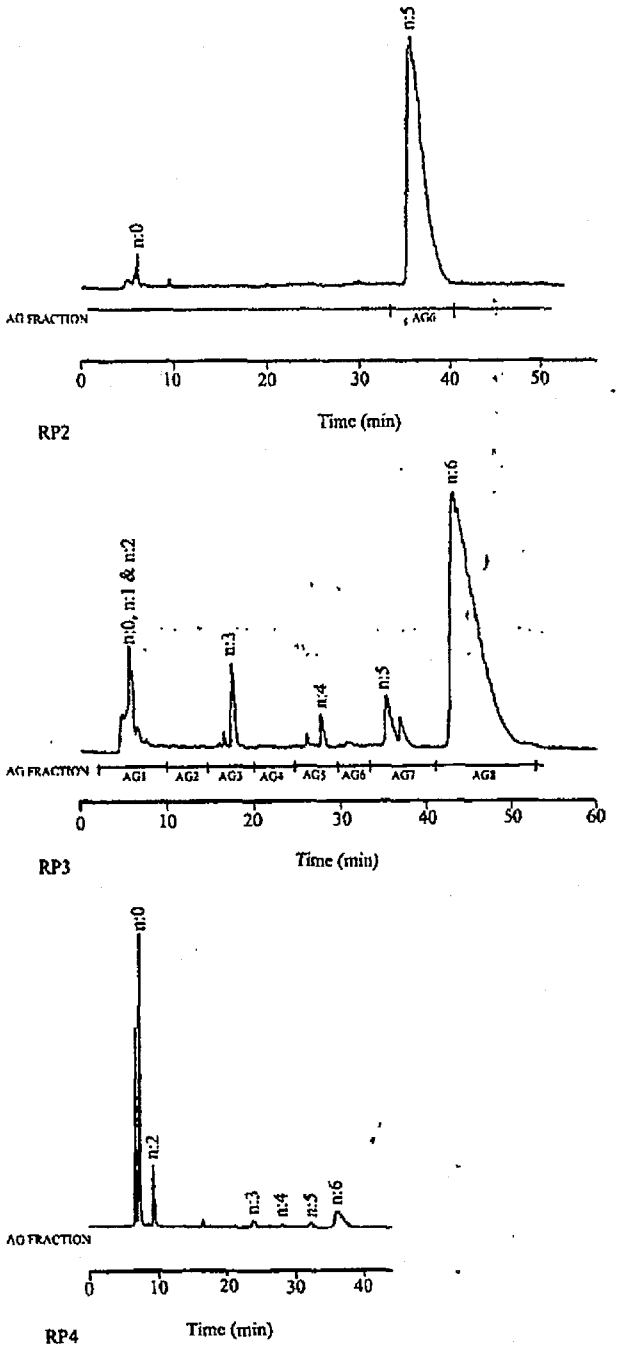

Fig. 4. Semi-preparative Ag–HPLC chromatogram of the bioactive RP–HPLC fractions RP2, RP3 and RP4. Chromatographic conditions: Chrompack Chromosphere, silver C18 semi-preparative column, using the multi-solvent system shown in Table 3, and a flow rate of 1.5 mL/min with 1 mg of each applied. Light scattering detector settings: ESV +70 °C, gas pressure 14 psi. The identified peaks represent the fatty acid methyl esters, with varying degree of unsaturation indicated (*n*, denoting the number of double bonds).

representative chromatogram of each in Fig. 4. The assignment of the degree of unsaturation was obtained by comparing the retention times with commercial standards.

The fraction RP2 (300 mg) was applied to the Ag–HPLC and showed one predominant peak (Fig. 4). This peak, which contained five double bonds, was collected and coded "$AG6_1$"

Table 5
Percent inhibition of the production of LO metabolites by AG fractions

Code	Dilution	Inhibition (%)[1]			
		6-*trans* LTB_4	6-*trans*,12-*epi* LTB_4	LTB_4	5-HETE
AG1	1:100	41±13	34±10	8±6	29±5
	1:1000	28±16	27±21	29±17	23±14
	1:10,000	25±17	26±13	30±16	23±15
AG3	1:100	65±8	59±9	51±9	54±9
	1:1000	3±15	2±13	0±9	NT[2]
	1:10,000	10±19	11±21	0±14	20±14
AG5	1:100	82±13	70±12	64±20	71±15
	1:1000	0±25	0±20	4±11	10±3
	1:10,000	0±18	0±23	0±8	6±5
AG6	1:100	48±14	46±13	47±9	44±5
	1:1000	0±5	0±3	11±12	8±2
	1:10,000	0±15	0±18	2±4	11±17
AG7	1:100	25±10	21±10	3±4	40±5
	1:1000	0±15	0±10	1±7	4±7
	1:10,000	0±12	0±13	0±5	0±11
AG8	1:100	74±9	77±1	91±2	72±0[3]
	1:1000	41±10	35±14	37±13	23±14
	1:10,000	0±18	0±17	15±10	14±18
$AG6_1$	1:100	59±3	56±4	27±16	50±4
	1:1000	22±22	22±17	16±25	39±25
	1:10,000	16±11	19±12	4±12	17±2
EPA	1:100	72±10	80±11	39±14	44±12
	1:1000	32±7	32±6	16±6	24±8
	1:10,000	31±17	31±16	110±27	13±15

[1]Control values were, typically (ng/10^6 cells, mean±s.d, n=4): 6-*trans* LTB_4, 39.9±5.0; 6-*trans*,12-*epi* LTB_4, 39.0±5.8; LTB_4, 20.1±1.7; 5-HETE, 134±8.6. The activity of the test samples is expressed as the % inhibition of control production of LO metabolites (mean±s.d, n=4). [2]NT (not tested). [3]n=1 replicate.

and was analysed for bioactivity by the *in vitro* assay. Fraction RP3 (387 mg) was applied to the system, and the fractions were collected according to the established time windows as shown in Fig. 4. Fractions 2 and 4 contained no material and therefore were not further analysed. The remaining fractions, coded "AG1", "AG3", "AG5", "AG6", "AG7" and "AG8" were screened for bioactivity by the *in vitro* assay. Finally RP4 was subjected to the Ag–HPLC and a similar chromatogram to RP3 was produced (Fig. 4).

3.7. Leukotriene in vitro assay of Ag–HPLC Fractions

The *in vitro* assay was performed on fractions $AG6_1$, AG1, AG3, AG5, AG6, AG7, and AG8 in addition to the eicosapentaenoic acid (EPA) commercial standard (Table 5).

Table 6
Preliminary identification by GC of the major components in fractions AG5 and AG6

Retention time (min)	Compound	% of total fraction	
		AG5	AG6
27.34	Eicosapentaenoic acid	0.1	–
26.00	Arachidonic acid	8.3	0.2
29.85	Unknown C21:5	0.1	–
27.03	Unknown C20:4	67.9	73.0
24.48	Unknown C19:4	2.8	1.0
23.47	Unknown C18:4	7.4	9.9

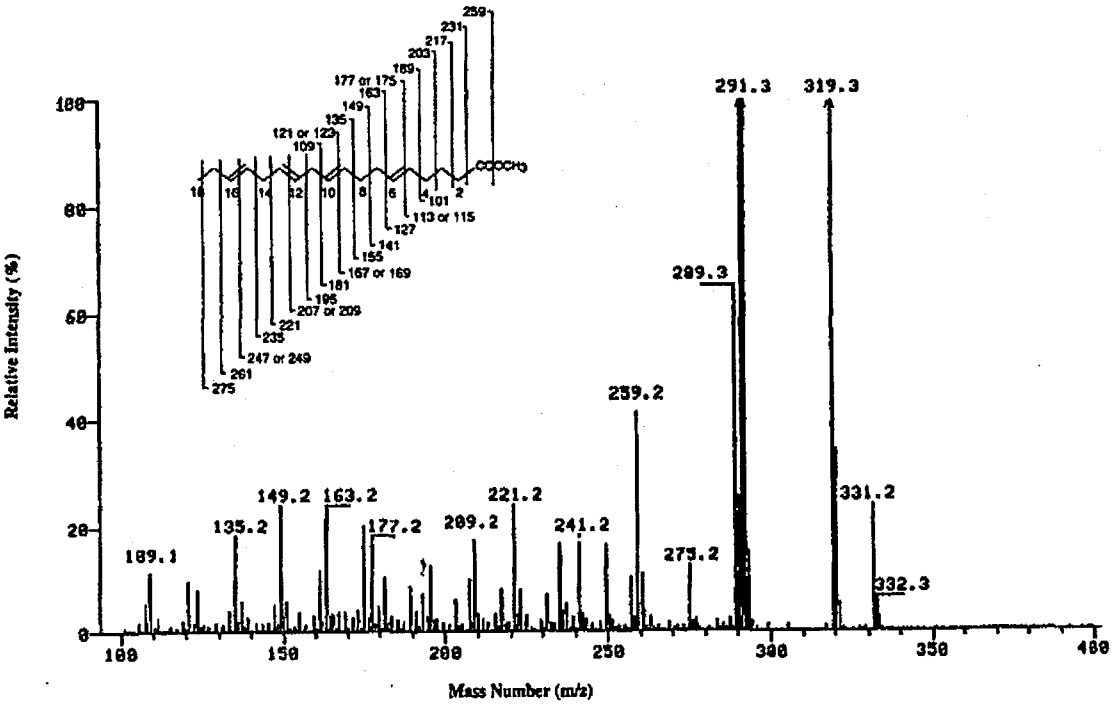

Fig. 5. Chemical ionisation mass spectrum of the methyl ester of the C18:4 FA in bioactive fraction AG5. Operating parameters: methane reactant gas, scan range 50–500 Da at 0.6 s/scan, interface temperature 280 °C, injector temperature 250 °C, and the ion source temperature 180 °C.

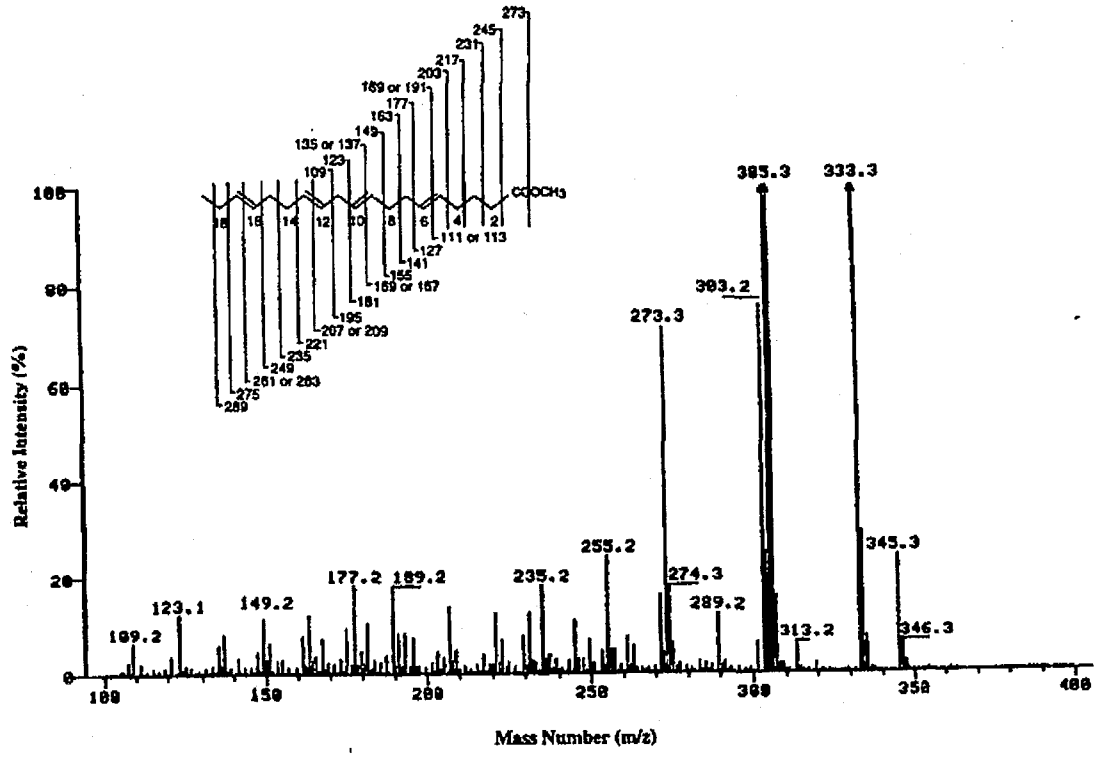

Fig. 6. Chemical ionisation mass spectrum of the methyl ester of the C19:4 FA in bioactive fraction AG5. Operating parameters: methane reactant gas, scan range 50–500 Da at 0.6 s/scan, interface temperature 280 °C, injector temperature 250 °C, and the ion source temperature 180 °C.

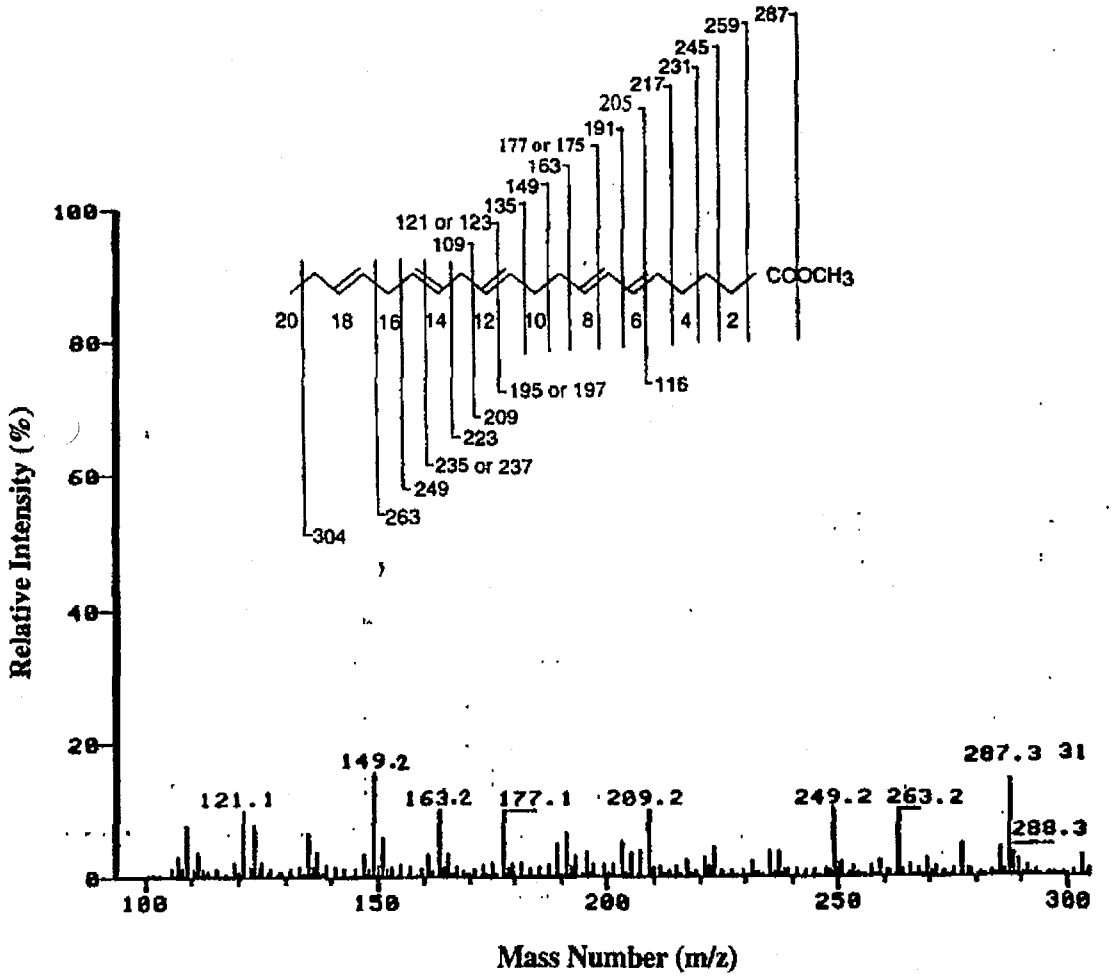

Fig. 7. Chemical ionisation mass spectrum of the methyl ester of the C20:4 FA in bioactive fraction AG5. Operating parameters: methane reactant gas, scan range 50–500 Da at 0.6 s/scan, interface temperature 280 °C, injector temperature 250 °C, and the ion source temperature 180 °C.

The results demonstrate that fractions AG5 and AG8 had the highest activities (1:100 dilutions).

3.8. Analysis of bioactive Ag–HPLC fractions by GC

Fractions which were shown to be bioactive from the *in vitro* neutrophil assay were analysed by GC, which indicated fractions AG1 and AG3 contained known unsaturated FFA including EPA. Fractions AG5 and AG6 however contained several novel compounds, and their percentage abundance are shown in Table 6. Fraction AG5 contained four unknown compounds in addition to EPA and AA. Fraction AG6 contained three unknown compounds and a trace of AA. Fraction AG8 was found by GC analysis to exclusively contain docosahexaenoic acid (DHA). Consequently, fractions AG5 and AG6 were further characterised by GC-MS.

3.9. GC-MS of active fractions from Ag–HPLC separation

The GC-MS analyses of fractions AG5 and AG6 were performed in both the CI and EI modes. Figs. 5–8 show the PICI mass spectra of the novel compounds and the fragmentation proposed to support the structural assignments. While neutral loss and impact fragmentation from protonated parents is a less energetic approach to EI of the same analytes, polyunsaturated compounds still show some propensity for rearrangement. This novel use of full scan PICI mass spectrometry was of benefit in that it provided for bioprospective examination of fractions on the basis of carbon number, degree of unsaturation and unstable substitution while also providing a population of intact and diagnostic high mass fragments that would be atypical in EI mode. Interpolation of structure from the similar fragmentation of known standards, such as EPA, AA and DHA, that contained common structural elements allowed the tentative assignment of structure that were then able to be confirmed by the same technique against known reference compounds available by independent routes.

The CI-MS of C18:4 in bioactive fraction AG5 is shown in Fig. 5. The proposed structure of this compound as the methyl ester is indicated, with the fragmentation shown for $M^{+}+H=291$ and a molecular formula of $C_{19}H_{30}O_2$. The compound was identified as having the bond positions of 5, 9, 12 and 15. The

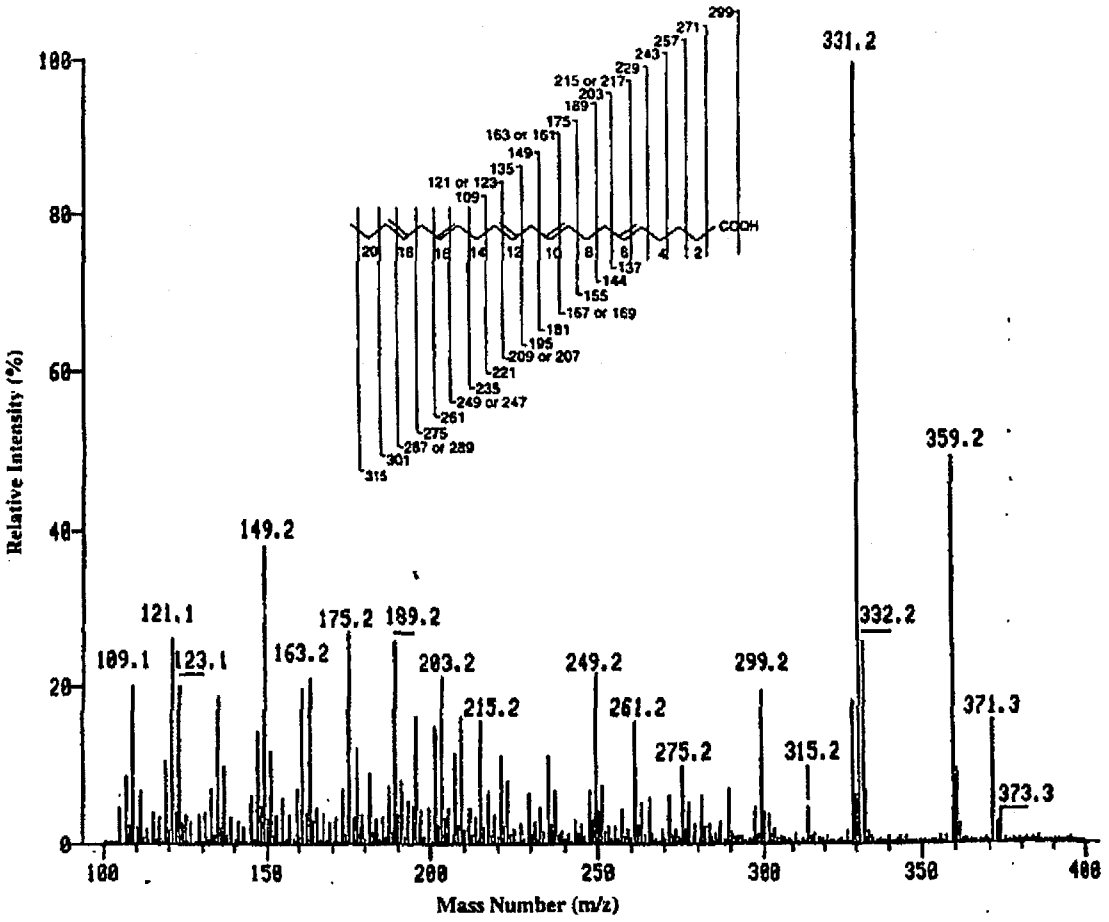

Fig. 8. Chemical ionisation mass spectrum of the methyl ester of the C21:5 FA in bioactive fraction AG5. Operating parameters: methane reactant gas, scan range 50–500 Da at 0.6 s/scan, interface temperature 280 °C, injector temperature 250 °C, and the ion source temperature 180 °C.

unsaturated centre C5/C6 position was indicated by the gap between m/z 189 and 163, positions C9/C10 by the gap between m/z 135 and 109, positions C12/C13 by the gap between m/z 221 and 195, positions C15/C16 by the gap between m/z 261 and 235.

The CI-MS of C19:4 in bioactive fraction AG5 is shown in Fig. 6 and the structure proposed to be consistent with the methyl ester with M^++H=305 and $C_{20}H_{32}O_2$. The compound was identified as having the bond positions of 5, 9, 12 and 16. With an additional methylene group in the chain, the unsaturated centre C5/C6 position was indicated by the gap between m/z 203 and 177, positions C9/C10 by the gap between m/z 149 and 123, positions C12/C13 by the gap between m/z 221 and 195, positions C16/C17 by the gap between m/z 275 and 249.

The CI-MS of C20:4 in bioactive fraction AG5 is shown in Fig. 7. Fragmentation supports the assignment of the methyl ester with M^++H=319 and molecular formula $C_{21}H_{34}O_2$. The compound was identified as having the bond positions of 7, 11, 14 and 17. The unsaturated non-skipped double bond centre C7/C8 position was assigned on the basis of the strong allylic fragment at m/z 149 and the fragment at m/z 163. Further assignment for the C7 double bond produced complex fragmentation resulting from bond migration for the isolated carbon double bond. Comparison of the spectra of the methyl esters of AA and EPA with that of the FAME at retention peak 27.03 min supports the assignment of this PUFA as 7, 11, 14, 17-eicosatetraenoic acid.

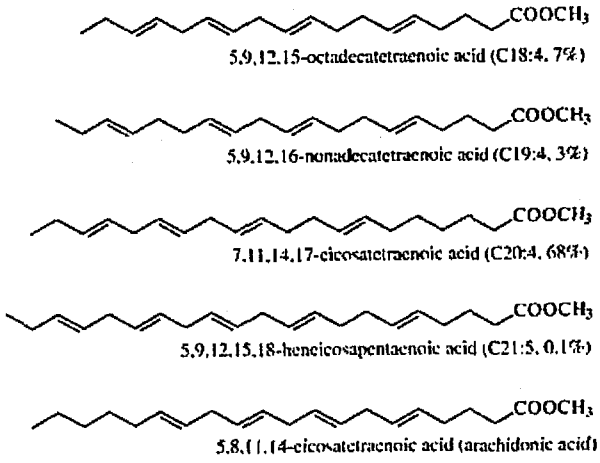

Fig. 9. The structures of ω-6 AA, and the novel bioactive ω-3 FAME from AG5 (as identified by GC-MS) and their percent concentration of the AG5 fraction. The novel compounds were identified as C18:4, C19:4, C20:4 and C21:5, and are an homologous series where the second double bond is separated from the first by more than one methylene group.

The CI-MS of C21:5 in bioactive fraction AG5 is shown in Fig. 8 and the data support the assignment as the methyl ester with M^++H=331 and molecular formula $C_{22}H_{34}O_2$. The compound was identified as having the bond positions of 5, 9, 12, 15 and 18. The unsaturated centre C5/C6 position was identified by the gap between m/z 229 and 203, positions C9/C10 by the gap between m/z 175 and 149, positions C12/C13 by the gap between m/z 135 and 109, positions C15/C16 by the gap between m/z 261 and 235, and the position C18/C19 by the gap between m/z 275 and 301.

The novel compounds identified were all ω-3 FAME in an homologous series (Fig. 9). The FAME showed structural similarity in the occurrence of the second double bond from the proximal end (carboxyl end) of each acid being separated from the first by more than one methylene group. The compounds were identified as 5, 9, 12, 15-octadecatetraenoic acid (C18:4); 5, 9, 12, 16-nonadecatetraenoic acid (C19:4); 7, 11, 14, 17-eicosatetraenoic acid (C20:4) and 5, 9, 12, 15, 18-heneicosapentaenoic acid (C21:5). The predominant compound was the C20:4 fatty acid which is a structural isomer to AA.

4. Discussion

The potential AI activity of lipids isolated from the green-lipped mussel of New Zealand, *P. canaliculus*, was investigated in this study. The tartaric acid-stabilised freeze dried powder of the mussel was subjected to supercritical-CO_2 fluid treatment, and the resulting oil was fractionated for its lipids using standard methodology. The SFE process utilises liquefied CO_2 to avoid the use of any chemical solvents that could create health or acceptability concerns for human applications.

The FFA class was purified from the mussel extract and then rigorously fractionated in order to isolate and identify the bioactive constituents. The group purification was carried out by two means, namely column chromatography and NP–HPLC. The column chromatography was performed to remove the highly polar mussel lipids and offered a reproducible means of separation. However, large quantities of solvents were consumed. Group purification by NP–HPLC provided a fast and efficient separation technique that was comparable to conventional open column technique. While the entire FFA class was not completely resolved by the NP–HPLC, the unsaturated FFA portion was relatively free from other lipid classes. Fractionation of the FFA group of the mussel extract utilised two complementary systems, namely RP–HPLC and Ag–HPLC. The RP–HPLC served as a preliminary fractionation stage in which the elution sequence of the fatty acids was governed by chain length and the presence of double bonds. The Ag–HPLC was carried out using $AgNO_3$–impregnated silica columns, which offer a sensitive means of separation. In the presence of silver ions, the separation was determined by the number of double bonds in the FAME, with FAME with *cis* double bonds eluting slower than those with double bonds in the *trans* configuration (Nikolova-Damyanova, 1992).

The FFA fractions obtained from the RP procedures were subjected to a proven *in vitro* assay for AI activity that uses a rapid and sensitive approach for the quantification of leukotrienes released by stimulated neutrophils (Cleland et al., 1990). The leukotriene assay monitors the production of four of the LO pathway products, namely LTB_4, 6-*trans* LTB_4, 6-*trans* 12 *epi* LTB_4, and 5-HETE. The formation of these LO products was significantly reduced by the *P. canaliculus* FFA fractions. The RP–HPLC fractions (more specifically fractions RP2 to RP5) demonstrated higher bioactivity than the entire FFA class.

Analysis by GC of the bioactive fractions was employed to ascertain whether any of the FFA fractions contained novel compounds. It was found that three of the bioactive RP–HPLC fractions, namely RP2, RP3 and RP4, contained unusual fatty acids. These fractions were carried through to the subsequent purification stage of Ag–HPLC. The results of the *in vitro* analysis of the Ag–HPLC fractions showed that fraction AG8 had high bioactivity and was identified by GC as 4, 7, 10, 13, 16, 19-docosahexaenoic acid (i.e. DHA), a known long chain ω-3 PUFA. The AI activity of DHA is believed to be due to a substrate substitution effect which results in a reduction of the relative concentration of AA, thereby diminishing the production of pro-inflammatory eicosanoids (Calder, 2006).

It has been reported that other bivalves which do not possess the marked AI activity of *P. canaliculus*, contain considerable DHA in their lipid fraction (Ackman, 1990; Couch et al., 1982). Consequently, it is possible that the AI activity of *P. canaliculus* is not due entirely to the presence of DHA, but also to other PUFA. Indeed, other Ag–HPLC fractions exhibited marked bioactivity in the *in vitro* assay, i.e. fractions AG5 and AG6. Analysis by GC of fractions AG5 and AG6 showed that they contained several novel compounds. These novel compounds were identified by GC-MS as four ω-3 PUFA compounds, namely C18:4, C19:4, C20:4 and C21:5. The GC-MS was performed in the EI and CI modes. In the EI mode, the long chain carboxylic acids and their esters undergo considerable skeletal rearrangement and bond migration. Therefore, it is not possible to determine the exact position of the double bonds in these long chain unsaturated compounds from the EI mass spectra. This novel application of positive ion chemical ionisation mass spectrometry provided data that was truly complimentary to the more energetic impact technique both because it was lower in energy and diagnostic fragments were sourced from protonated parents thereby altering the thermodynamics influencing their formation. Using PICI in a bioprospective approach against external standards allowed assignment of the bond positions in the bioactive compounds of fractions AG5 and AG6. The structures of the unusual FFA of *P. canaliculus* were found to be an homologous series, and a possible AI mechanism can be inferred from a comparison of these structures with AA.

The inflammatory precursor AA is an ω-6 PUFA of 20 carbons in length and has 4 unsaturated double bonds (positions 5, 8, 11 and 14) with each double bond being separated by one methylene group. The predominant bioactive PUFA of *P. canaliculus* identified in this study is similar to AA in that it also possesses 20 carbons with four double bonds. However, the first double bond is located at the seventh position, and the second double bond is interrupted from the first by two methylene groups resulting in the double bonds at positions 7, 11, 14 and 17. A similar pattern is shown for the other three novel compounds identified, whereby the second double bond is separated from the

first by more than one methylene group. The interrupted bond positioning of these structural analogues of AA may account for their AI behaviour, by competitively inhibiting the active site of enzymes which use AA as a substrate, i.e., LO and COX, thereby reducing the production of leukotriene (LT) and prostaglandin (PG) metabolites.

We have previously demonstrated strong inhibition of the LT and PG metabolites of AA metabolism, as well as production of alternate LT and PG metabolites, for a variety of PUFA found in Lyprinol® (McPhee et al., 2001; McPhee et al., 2007). It is not yet known whether the novel homologous series of ω-3 PUFA isolated in this study are substrates for the AA metabolising enzymes; nevertheless, these compounds might not be converted to the endogenous eicosanoids, and consequently the inflammatory processes promoted by increased LT and PG levels would be diminished. In addition, inhibition of both COX and LO may also reduce the incidence of the side effects that occur when only PG production is inhibited (Charlier and Michaux, 2003).

In this study we isolated four potentially AI ω-PUFA from *P. canaliculus* using various chromatography techniques. The compounds identified are biologically significant as AI agents, and are structural analogues forming an ω-3 PUFA homologous series. The structure proposed for the most prominent PUFA has double bonds at alternative positions to AA, the inflammatory pathway precursor. This implies that this prominent C20:4 and its homologues may act as inhibitors of LT and PG metabolite production in the inflammation pathways.

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Anti-cyclooxygenase effects of lipid extracts from the New Zealand green-lipped mussel, *Perna canaliculus*

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Abstract

Total lipid extracts of *P. canaliculus* (a bivalve marine mollusc native to New Zealand, commonly called the green-lipped mussel) and *Mytilus edulis* (commonly called the common blue mussel) moderately inhibited ovine COX-1 and COX-2 pure enzymes *in vitro*. The inhibition was increased after the mussel extracts were saponified by KOH hydrolysis. Protease- and protease–lipase-hydrolysed lipid extracts of *P. canaliculus* exhibited similarly strong COX inhibition as the KOH-hydrolysed extract. Lyprinol® (a commercial extract from *P. canaliculus*) also exhibited strong inhibition of both COX isoforms, an effect that was increased 10-fold upon subsequent hydrolysis. In contrast, fish oil was not as anti-COX active as Lyprinol. The Lyprinol free fatty acid fraction, and to a lesser extent the Lyprinol triglyceride fraction, were the only lipid classes of Lyprinol to exhibit strong inhibition of the COX isoforms. The purified PUFA extracts were all bioactive, potently inhibiting COX-1 and COX-2. Incubation of Lyprinol in the absence of exogenous arachidonic acid (AA) showed the appearance of alternate prostaglandin metabolites, confirming Lyprinol PUFA as a competitive substrate inhibitor of AA metabolism.

Keywords: Anti-inflammatory; Cyclooxygenase; Lyprinol®; *Perna canaliculus*; Polyunsaturated fatty acids

1. Introduction

Cyclooxygenase (COX) are lipid metabolising enzymes that catalyse the oxygenation of polyunsaturated fatty acids (PUFA), preferably arachidonic acid (AA), to form the prostanoids, which are potent cell-signalling molecules associated with the initiation, maintenance and resolution of inflammatory processes (Charlier and Michaux, 2003). Selective modulation of the many prostanoids has important therapeutic potential for the treatment of inflammation and inflammatory conditions such as rheumatoid arthritis. Traditional drug treatments for relieving the pain and swelling of inflammation include aspirin, indomethacin and other non-steroidal anti-inflammatory drugs (NSAIDs). Unfortunately, NSAIDs are also noted for undesirable gastrointestinal side effects related to their use (Parente and Perretti, 2003). For this reason, selective COX-2 inhibitors (the 'Coxibs') have come to attention in recent years. As coxibs are increasingly used in clinical practice, it appears that they too exhibit side effects, most notably in relation to cardiovascular disturbances. As a result, medical researchers are looking for safer, more efficacious alternatives to both the traditional NSAIDs and the more recent COX-2 selective inhibitors.

One natural alternative for the treatment of inflammation is the New Zealand green-lipped mussel, *Perna canaliculus* (Bivalvia: Mytilidae). This marine mollusc has demonstrated gastroprotective, antihistaminic, antioxidant, anticytokine, anti-inflammatory and antiarthritic properties (Gibson et al., 1980; Rainsford and Whitehouse, 1980; Kosuge et al., 1986; Whitehouse et al., 1997; Mani et al., 1998; Tan and Berridge, 2000); with bioactive principles reportedly residing in the protein, polysaccharide and lipid fractions of the mussel (Miller and

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Ormrod, 1980; Couch et al., 1982; Miller et al., 1993; Mani et al., 1998; Tan and Berridge, 2000). Commercial preparations of *P. canaliculus* include the stabilised freeze-dried anti-inflammatory mussel powder, Seatone® (Kosuge and Sugiyama, 1989), and Lyprinol® which is the mussel "oil" obtained by supercritical fluid extraction of the stabilised mussel powder using liquefied carbon dioxide (Macrides and Kalafatis, 2000). The lipid-rich Lyprinol extract shows significant anti-inflammatory activity when given both therapeutically and prophylactically po to Wistar and Dark Agouti rats developing either antigen-induced polyarthritis or collagen (II)-induced autoallergic arthritis, with ED_{50} values ≤ 15 mg/kg, compared with naproxen $ED_{50} \geq 25$ mg/kg or with other therapeutic oils (flaxseed, evening primrose, fish) $ED_{50} \geq 1800$ mg/kg (Whitehouse et al., 1997). Lyprinol exhibits little or no efficacy in acute irritation assays (carrageenin, kaolin, histamine); but in contrast to classical NSAIDs, Lyprinol is non-gastrotoxic in diseased-stressed rats at 300 mg/kg po, and has no effect on *ex vivo* platelet aggregation triggered by arachidonate or ADP, either in rats (50 mg/kg) or humans (150 mg/kg) (Whitehouse et al., 1997). *In vitro* biochemical testing of Lyprinol has been undertaken to more fully understand the anti-inflammatory mechanism of the lipid extract, and much of this work has focused on the eicosanoid biochemical pathways found in inflammatory cells. Inhibition of leukotriene-B_4 production by Lyprinol has been observed in calcium and ionophore-stimulated human neutrophils (Whitehouse et al., 1997) and also in interleukin-4-induced human monocytes (Dugas, 2000). Inhibition of prostaglandin-E_2 production in activated human macrophages has also been observed (Whitehouse et al., 1997). Lyprinol, therefore, appears to exert some effect on the arachidonic acid cascade.

In this study, the anti-inflammatory properties of various lipid extracts of the New Zealand green-lipped mussel *P. canaliculus* was studied *in vitro* in relation to arachidonic acid metabolism by COX enzymes. Lyprinol and its component ω3 PUFAs were also tested for anti-COX activity, and this activity was compared to the common blue mussel *Mytilus edulis*, to fish oil, and to the known NSAID indomethacin. The production of COX metabolites from Lyprinol was also investigated by gas chromatography mass spectroscopy (GCMS).

2. Materials and methods

2.1. Chemicals

Pure standards of AA, various PUFA, prostaglandins and purified COX enzymes were obtained from Cayman Chemical Company (Ann Arbor, MI, USA). Indomethacin, 2,7-dichlorofluorescein, 2,7-dichlorofluorescein diacetate, hematin and phenol were obtained from Sigma Chemical Company (St Louis, MO, USA). Lipid standard 18-5A was obtained from NuCheck (Minneapolis, MI, USA). Neutrase® 0.8L (endoprotease) and Novozym® 871L (*sn*-1, *sn*-3-specific lipase) were donated by Novo Nordisk (Bagsvaerd, Denmark). Silica gel for column chromatography (Kieselgel 60, 230–400 mesh), thin layer chromatography (TLC) plates (Kieselgel 60 G, 5–40 gm), and pre-prepared TLC plates (Kieselgel 60 F254 nano DC) were sourced from Merck (Darmstadt, Germany). Silica Sep-Pak cartridges and 0.45 μm polytetrafluoroethylene (PTFE) syringe filters were obtained from Waters Corporation (Milford, MA, USA). High performance liquid chromatography (HPLC)-grade methanol and other solvents used for lipid extraction and analysis were supplied by Selby Biolab (Clayton, Victoria, Australia). All other general laboratory chemicals and reagents were of analytical quality and obtained through Merck (Darmstadt, Germany).

2.2. Lipid test samples

P. canaliculus and *M. edulis* mussel samples were collected from Hallam Cove, New Zealand, March 2000, by Dr Henry Kaspar (Cawthron Institute, New Zealand). The whole body mussel meat was refrigerated (4 °C) then processed by freeze-drying within 24 h of collection. Lyprinol® was supplied by McFarlane Laboratories (Melbourne, Victoria, Australia). Blackmores Fish Oil 1000® was purchased from a Melbourne pharmacy. Lyprinol, fish oil, and a variety of purified PUFA were dissolved in HPLC-grade methanol ready for biochemical analysis.

2.3. Lipid extraction

Total lipid extracts of the freeze-dried mussel meat of *P. canaliculus* and *M. edulis* were obtained using a standard chloroform–methanol extraction procedure (Bligh and Dyer, 1959). Three grams of freeze-dried meat was used in each respective extraction (from approximately 20 combined individuals). The extracted lipid material was dissolved in HPLC-grade methanol ready for biochemical analysis, or taken for further processing (saponification).

2.4. Hydrolysis of total lipid extracts

P. canaliculus and *M. edulis* total lipid extracts were saponified by KOH hydrolysis according to the method of Christie (1989). Pyrogallol (5%) was included in the hydrolysis mixture as an antioxidant. Saponified extracts containing the total free fatty acid fraction were dissolved in HPLC-grade methanol ready for biochemical analysis.

2.5. Enzyme assisted extractions of P. canaliculus

The protease *P. canaliculus* lipid extract (P) was prepared by addition of Neutrase® 0.8 L (5 mL) to freshly thawed whole mussel in shell (1.5 kg, purchased from a local Melbourne fishmonger) with incubation (2 h, 40 °C). The protease–lipase *P. canaliculus* lipid extract (PL) was prepared by digestion with Neutrase, followed by cleavage of esterified fatty acids with Novozym 871 L® (0.3 mL of lipase added to 620 mL of liquid mixture previously treated with neutrase) with incubation (3 h, 23 °C). Lipids were isolated from the respective aqueous digestions by freeze-drying followed by solvent extraction

using diethyl ether. Silica gel TLC analysis was performed on the extracts utilising a solvent system of hexane–ether–acetic acid (70:30:2 v/v/v) and visualised by spraying with phosphomolybdic acid before charring at 100 °C for 5 min. The lipid extracts were evaporated to dryness and each re-dissolved in HPLC-grade methanol ready for biochemical analysis.

2.6. Hydrolysis of Lyprinol

Lyprinol was saponified by KOH hydrolysis according to the method of Christie (1989). Pyrogallol (5%) was included in the hydrolysis mixture as an antioxidant. Saponified Lyprinol containing the total free fatty acid fraction was dissolved in HPLC-grade methanol ready for biochemical analysis.

2.7. Preparation of Lyprinol lipid classes

Separation of Lyprinol (1.07 g) into its constituent lipid classes was achieved using open column chromatography on a stationary phase of silica gel, and a polarity gradient of methyl-*tert*-butyl ether (MTBE) in hexane. Column fractions of sterol esters, triglycerides, free fatty acids, sterols and phospholipids were combined to give one fraction for each lipid class. Each lipid class was evaporated to dryness and weighed to obtain percentage composition. The purity of the separated lipid classes was determined by silica gel TLC utilising a solvent system of hexane-MTBE-acetic acid (80:20:2 v/v/v), and visualised by spraying with phosphomolybdic acid before charring at 100 °C for 5 min. The separated Lyprinol lipid classes were diluted in HPLC-grade methanol ready for biochemical analysis, or taken for further processing (methylation).

2.8. Methylation of Lyprinol lipid classes

The individual lipid classes were hydrolysed with 3.8% KOH and converted to fatty acid methyl ester (FAME) by the addition of boron trifluoride. Separation of the FAME by double bond number was achieved using silver impregnated silica TLC plates and a solvent system of hexane-MTBE (40:60 v/v), and visualised at 254 nm after spraying with 2,7-dichlorofluorescein. The individual FAME bands were extracted from the silica twice with MTBE-hexane (1:1 v/v), filtered through a 45 μm PTFE filter, and evaporated to dryness.

Extracted residues were treated with an ethereal solution of diazomethane containing 10% by volume of methanol. The treated samples were evaporated to dryness under nitrogen at room temperature. Residues were reconstituted in ethyl acetate (200 μL) and the mixture was heated in a sealed tube at 80 °C for 20 min before being transferred to an autosampler vial prior to GCMS analysis. A second procedure for methylation involved treatment with *N*-trimethylsilyl-trifluoroacetamide (MSTFA, 35 μL, Pierce, Rockford, IL, USA) and toluene (65 μL).

2.9. GCMS analysis

Electron impact (EI)-GCMS was performed on a Hewlett Packard 6890 GC-5973 MSD with a 7683 injector (Palo Alto, USA). The GC was equipped with a BPX5 column (12 m×0.2 mm, 0.25 μm film thickness, SGE, Melbourne, Australia) and used helium as the carrier gas with a constant flow of 1 mL/min. The oven temperature was held at 75 °C for 2 min then increased at 30 °C/min to 300 °C with a final holding time of 9 min. Injections of 2 μL were pulsed splitless with a nominal head pressure of 60 kPa pulsed to 170 kPa for 1 min after injection. A scan range of 50–650 Da at 1.0 scan/s was used.

Fatty acids, sterols and their metabolites were identified by comparison of their EI mass spectra with those of authentic standards, where available. In the absence of suitable standards of the proposed metabolites, assignments were supported by response to methylating reagents, retention time and mass spectral data. Assignments were based on EI mass spectral fragmentation, on positive-ion chemical ionisation assigned molecular ions, and on common fragmentation pathways established for structurally analogous compounds.

2.10. Cyclooxygenase inhibition assay

The cyclooxygenase inhibition assay was performed according to a modified method of Larsen et al. (1996). The oxidation of leuco-dichlorofluorescein (1-DCF) in the presence of phenol by the hydroperoxide formed in the cyclooxygenase reaction can be used as a sensitive spectrophotometric assay for PGH-synthase activity (Larsen et al., 1996). Leuco-2,7-dichlorofluorescein diacetate (5 mg) was hydrolysed at room temperature in 1 M NaOH (50 μL) for 10 min, then 1 M HCl (30 μL) was added to neutralise excess NaOH before the resulting 1-DCF was diluted in 0.1 M Tris-buffer, pH 8. Cyclooxygenase enzyme (COX-1 or COX-2) was diluted in 0.1 M Tris-buffer, pH 8, so that a known aliquot gave an absorbance change of 0.05/min in the test reaction. Test samples (or the equivalent volume of methanol, 20 μL) were pre-incubated with enzyme at room temperature for 5 min in the presence of hematin. Premixed phenol, 1-DCF and arachidonic acid were added to the enzyme mixture to begin the reaction, and to give a final reaction mixture of arachidonic acid (50 μM), phenol (500 μM), 1-DCF (20 μM) and hematin (1 μM) in 1 mL final volume of 0.1 M Tris-buffer, pH 8. The reaction was recorded spectrophotometrically over 1 min at 502 nm. A blank reaction mixture was analysed in the spectrophotometer reference cell against each test reaction to account for any non-enzymatic activity attributed to the test sample. This blank consisted of the reaction mixture without the addition of enzyme. Lyprinol was investigated at multiple concentrations and exhibited 50% inhibition of the enzyme reaction at approximately 1 μg/mL (final concentration), therefore the other lipid samples were tested at this final concentration for comparison.

2.11. Cyclooxygenase metabolite analysis by GCMS

Cyclooxygenase enzyme (COX-1 or COX-2), phenol and hematin in 0.1 M Tris-buffer, pH 8 were pre-incubated at 37 °C for 2 min before addition of arachidonic acid [or alternatively Lyprinol] diluted in 0.1 M Tris-buffer, pH 8, to give a final

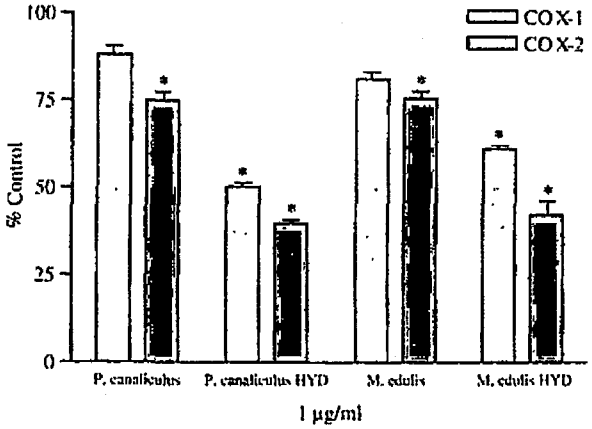

Fig. 1. Inhibition of COX-1 and COX-2 by *P. canaliculus* and *M. edulis* total lipid extracts at 1 µg/mL (mean±SEM, $n=3$). *Significantly different from methanol control based on one way ANOVA with Dunnett's post test ($p<0.05$). Hydrolysed lipid extract (HYD).

reaction mixture of phenol (500 µM), hematin (1 µM), arachidonic acid (80 µM) [or the equivalent weight of Lyprinol (24.36 µg/mL solution)], and 35 units of purified cyclooxygenase enzyme (COX-1 or COX-2). The reaction was run for 15 min and terminated with citric acid (0.23 M) to pH 3.5. The metabolites were then extracted with 5 mL of HPLC-grade chloroform. Each reaction was performed in triplicate and the extracted mixtures combined before separation by TLC to allow ample metabolite quantity for GCMS analysis.

Each extracted mixture was evaporated with nitrogen to approximately 200 µL and applied as a single band to a 10×20 cm silica gel (Kiesel gel 60) TLC plate. The metabolites were separated on a mobile phase of chloroform–methanol–acetic acid–water (90:8:1:0.8 v/v/v/v) and visualised by UV lamp at 254 nm, and the plate divided into five individual bands. Each band was scraped from the plate, extracted with 5 mL of chloroform-methanol (2:1 v/v) and centrifuged for 10 min to sediment the silica particles. Each fraction was filtered from the silica using a PTFE filter and washed through with 2 mL of chloroform before being evaporated under nitrogen. Extracted fractions were treated with an ethereal solution of diazomethane containing 10% by volume of methanol. The treated fractions were evaporated to dryness under nitrogen at room temperature. Fractions were reconstituted in ethyl acetate (200 µL) and the mixture was heated in a sealed tube at 80 °C for 20 min before being transferred to an autosampler vial prior to GCMS analysis. Electron impact (EI)-GCMS was performed as described above.

2.12. Statistical analysis

For the enzyme inhibition assay, the absorbance per min for each test was calculated over the first 60 s of the reaction. Each reaction was performed in triplicate and the results expressed as the mean±SEM ($n=3$) percentage of control, or percentage inhibition of control. Statistical significance between multiple sample groups was analysed by one-way ANOVA, with Dunnett's post hoc test when results were significant ($p<0.05$). Outlier results in the data were detected and removed using Grubbs outlier test.

3. Results

3.1. Inhibition of COX by P. canaliculus and M. edulis lipid extracts

Cyclooxygenase-1 and COX-2 were inhibited moderately by both *P. canaliculus* (COX-1, 12%; COX-2, 25%) and *M. edulis* (COX-1, 18%; COX-2, 24%) total lipid extracts at 1 µg/mL (Fig. 1). When these extracts were hydrolysed to their total free fatty acid fractions, significant inhibition of both COX isoforms was observed for *P. canaliculus* (COX-1, 49%; COX-2, 60%) and *M. edulis* (COX-1, 38%; COX-2, 57 %).

3.2. Inhibition of COX by P. canaliculus (P) and (PL) lipid extracts

At 1 µg/mL, the protease (COX-1, 57%; COX-2, 47%) and protease–lipase (COX-1, 67%; COX-2, 62%)-treated *P. canaliculus* exhibited strong, non-selective inhibition of both COX isoforms (Fig. 2). The (P) extract displayed significantly less inhibition overall than the (PL) extract ($p<0.05$). Analysis by TLC of the enzyme-treated extracts shows a decrease in triglycerides with concomitant increase in free fatty acids after combined protease–lipase treatment.

3.3. Inhibition of COX by Lyprinol and Lyprinol lipid classes

The CO_2 supercritical fluid lipid extract of *P. canaliculus*, Lyprinol, exhibited strong, concentration dependent inhibition of both COX-1 and COX-2, and was more inhibitory than the traditional NSAID COX inhibitor, indomethacin, at comparable concentrations (Fig. 3). Upon examination of the IC_{50} inhibitory concentration values (Fig. 4), hydrolysed Lyprinol exhibited approximately 10 times more inhibitory activity against COX than non-hydrolysed Lyprinol. Lyprinol (COX-1, 61%; COX-2,

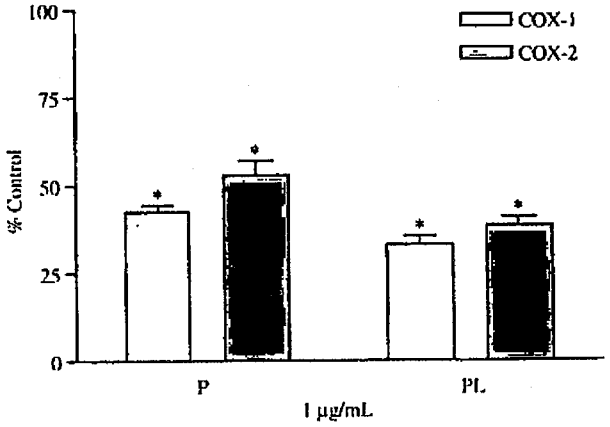

Fig. 2. Inhibition of COX-1 and COX-2 by enzyme-treated *Perna canaliculus* (mean±SEM, $n=3$). *Significantly different from methanol control based on one way ANOVA with Dunnett's post test ($p<0.05$). Protease extract (P); protease–lipase extract (PL).

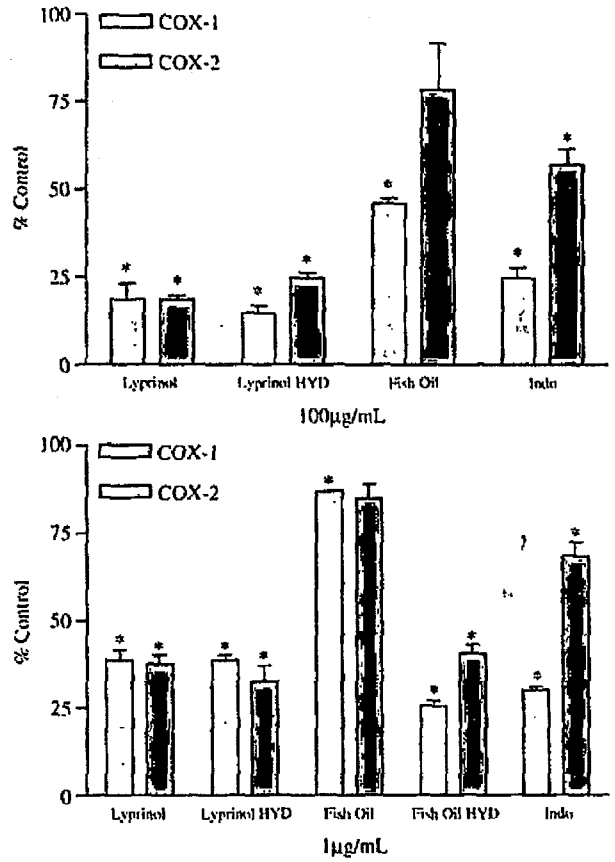

Fig. 3. Inhibition of COX-1 and COX-2 by Lyprinol, fish oil, and indomethacin at 100 and 1 µg/mL (mean±SEM, $n=3$). *Significantly different from methanol control based on one way ANOVA with Dunnett's post test ($p<0.05$). Hydrolysed (HYD), indomethacin (Indo).

62%) was also more effective at inhibiting COX than fish oil (COX-1, 13%; COX-2, 15%), although once fish oil was hydrolysed (COX-1, 74%; COX-2, 59%) it exhibited similar activity to that of Lyprinol and hydrolysed Lyprinol at 1 µg/mL.

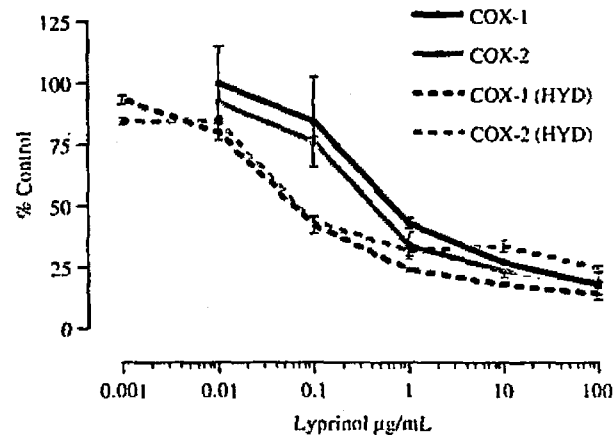

Fig. 4. Inhibition of COX-1 and COX-2 by different concentrations of unhydrolysed and hydrolysed Lyprinol (mean±SEM, $n=3$). IC_{50} values for Lyprinol were 0.7 µg/mL for COX-1 and 0.45 µg/mL for COX-2. IC_{50} values for hydrolysed (HYD) Lyprinol were 0.07 µg/mL for COX-1 and COX-2, respectively.

Table 1
Lipid class composition of Lyprinol

Lyprinol lipid class	Solvent elution (% MTBE in hexane)	Total Lyprinol (%)
Sterol esters	1–5	5.8
Triglycerides	5–10	26.4
Free fatty acids	10–25	53.7
Sterols	25–50	8.0
Phospholipids (polar lipids)	50–100*	6.1

*Includes 100% methanol fraction (to remove all remaining polar material from the column).

Free fatty acids comprised 54% of the Lyprinol fraction, with triglycerides the next most abundant lipid class comprising 26% (Table 1). Phospholipids, sterol and sterol esters comprised approximately equal amounts of the remaining lipid fraction. The free fatty acid fraction from Lyprinol exhibited the highest inhibition of all the Lyprinol lipid classes, and inhibition was high for both COX-1 (78%) and COX-2 (70%) (Fig. 5). The triglyceride fraction exhibited approximately 50% inhibition of both isoforms (COX-1, 43%; COX-2, 52%). There was limited inhibition of COX by the other Lyprinol lipid classes (≤32%). In particular, there was no significant inhibition of COX-1 by the sterol ester, sterol and phospholipids fractions, but there was moderate inhibition of COX-2 by these lipid classes (22%, 23% and 32%, respectively).

The free fatty acid and triglyceride fractions of Lyprinol were found to be abundant in monounsaturated and polyunsaturated fatty acids (Table 2). Saturated fatty acids accounted for over 45% of the total lipid composition of both fractions. The commercially sourced eicosapentaenoic acid (EPA, COX-1, 92%; COX-2, 91%), stearidonic acid (SA, COX-1, 94%; COX-2, 85%), ω3-arachidonic acid (ω3-AA, COX-1, 95%; COX-2, 89%), ω6-arachidonic acid (ω6-AA, COX-1, 97%; COX-2, 85%) and α-linolenic acid (α-LNA, COX-1, 92%; COX-2, 85%) all exhibited strong inhibition of COX-1 and COX-2 (Fig.

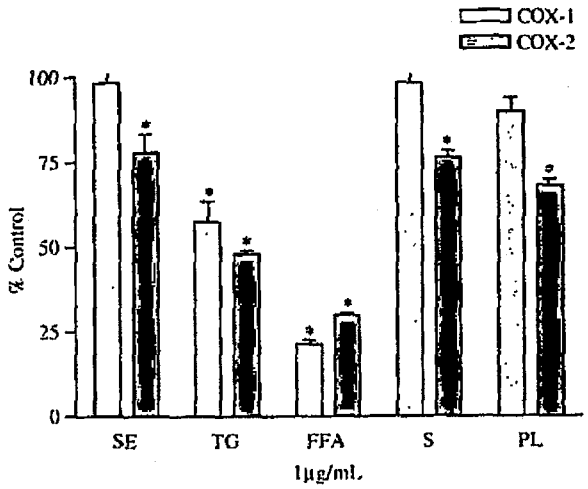

Fig. 5. Inhibition of COX-1 and COX-2 by Lyprinol lipid classes (mean±SEM, $n=3$).*Significantly different from methanol control based on one way ANOVA with Dunnett's post test ($p<0.05$). Sterol esters (SE); triglycerides (TG); free fatty acids (FFA); sterols (S); phospholipids (PL).

6). Adrenic acid (AdA, COX-1, 76%; COX-2, 75%) was slightly less inhibitory of the COX isozymes than the previously tested fatty acids. Docosahexaenoic acid (DHA, COX-1, 70%; COX-2, 85%) and docosapentaenoic acid (DPA, COX-1, 65%; COX-2, 95%), and especially eicosatrienoic acid (ETA, COX-1, 30%; COX-2, 77%) and docosatrienoic acid (DTA, COX-1, 51%; COX-2, 84%), exhibited less inhibition of COX-1, although inhibition of COX-2 was strong.

3.4. Cyclooxygenase metabolite analysis by GCMS

Incubation of ω6-AA with COX-1 resulted in production of prostaglandin products as indicated by GCMS analysis (Fig. 7). In relation to the fractions scraped from the TLC plate, fractions 3 and 4 contained multiple prostaglandin peaks, but because of the sample complexity these have not been identified conclusively. The presence of arachidonic acid in fraction 2 (Rt=9 min) indicated that not all the substrate was utilised in the enzyme reaction. The COX-2 reaction on arachidonic acid displayed a similar metabolite profile to the COX-1 reaction, however it appeared less abundant in prostaglandin product (Fig. 8).

Incubation of Lyprinol with COX-1 (Fig. 9) and COX-2 (Fig. 10) also indicated the presence of prostaglandin metabolites in fractions 3 and 4, despite the fact there was no addition of exogenous arachidonic acid. The prostaglandin metabolites were less evident in the COX-2 Lyprinol reaction compared to the COX-1 Lyprinol reaction. In addition, fraction 2 from the Lyprinol reactions (COX-1 and COX-2) indicated non-arachidonate-derived fatty acid esters eluting in the customary arachidonic acid ester region (Rt range 8–9 min).

Table 2
Fatty acid composition of the FFA and TG fractions from Lyprinol

Fatty acid	Percentage FAME in total fraction	
	FFA fraction	TG fraction
4,8,12-trimethyl 10:0		0.004
14:0	8.22	3.61
15:0	1.01	0.78
16:0	38.28	31.18
16:0/17:0		1.25
14-methyl 16:0		0.004
17:0		2.09
18:0	4.8	7.95
20:0	0.07	
20:0/20:1ω-9*		12.59
16:1ω-7		0.09
18:1	9.13	12.34
18:1ω-9/18:2*		0.08
20:1 (2 isomers)	4.75	
20:1ω-9/20:2ω-7*		0.05
16:2ω-4	1.82	0.02
16:2ω-6	0.56	0.14
17:2		0.005
18:2	7.64	0.33
20:2	0.52	
20:2ω-9		1.16
20:2/20:3*	1.62	
21:2		0.002
22:2	0.13	1.81
22:2/22:3*	0.38	
16:3	0.15	0.78
18:3ω-3	5.5	1.67
18:3ω-6	0.06	0.32
18:3ω-3/18:4ω-3*	1.83	
20:3	4.13	0.80
22:3	1.64	0.55
18:4ω-3	0.04	1.27
20:4ω-3		0.26
20:4ω-6		0.56
22:4/22:5*		0.02
20:5ω-3	1.14	1.83
21:5	0.05	0.06
22:5	0.03	
22:6ω-3	0.38	0.05
22' fatty acids		0.24
TOTAL	94.15	83.90

*Unresolved peaks.

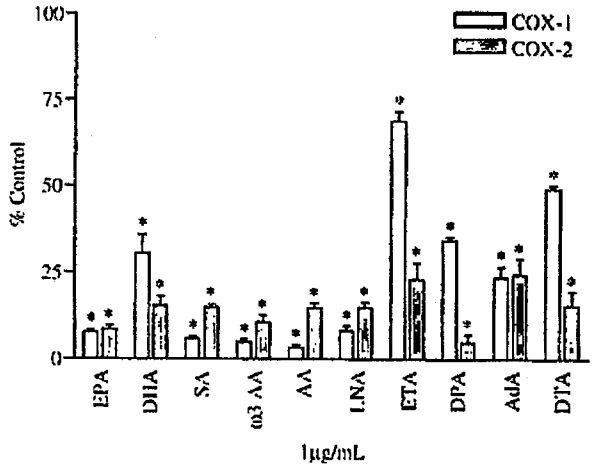

Fig. 6. Inhibition of COX-1 and COX-2 by PUFA from Lyprinol (mean±SEM, $n=3$). *Significantly different from methanol control based on one way ANOVA with Dunnett's post test ($p<0.05$). Eicosapentaenoic acid (EPA); docosahexaenoic acid (DHA); stearidonic acid (SA); ω3 arachidonic acid (ω3 AA); ω6 arachidonic acid (AA); linolenic acid (LNA); eicosatrienoic acid (ETA); docosapentaenoic acid (DPA); docosatetraenoic acid (AdA, adrenic acid); docosatrienoic acid (DTA).

4. Discussion

Marine organisms such as the bivalve mussels examined in this study contain an abundance of bioactive lipids. Bioactive lipids can include fatty acids, sphingolipids, phytosterols, diacylglycerols, diterpenes, sesquiterpenes and saponins; and many of these can influence human health and disease (Glaser and Lock, 1995; Huynh et al., 1997; Li and Sinclair, 2002). Mussels, like other marine organisms such as oily fish, are abundant in ω3 PUFA, particularly EPA and DHA (Joseph, 1982; King et al., 1990). Marine oils have been linked to alleviating the symptoms of inflammatory conditions, such as arthritis, skin disorders and asthma (Calder, 2001), as well as reducing certain risk factors of cardiovascular disease (Herold and Kinsella, 1986; Paganelli et al., 2001).

Lipid analysis of Hallam Cove *P. canaliculus* used in this study reveals a complex mixture of marine PUFA (comprising 45% of total lipid), including high concentrations of EPA (13%)

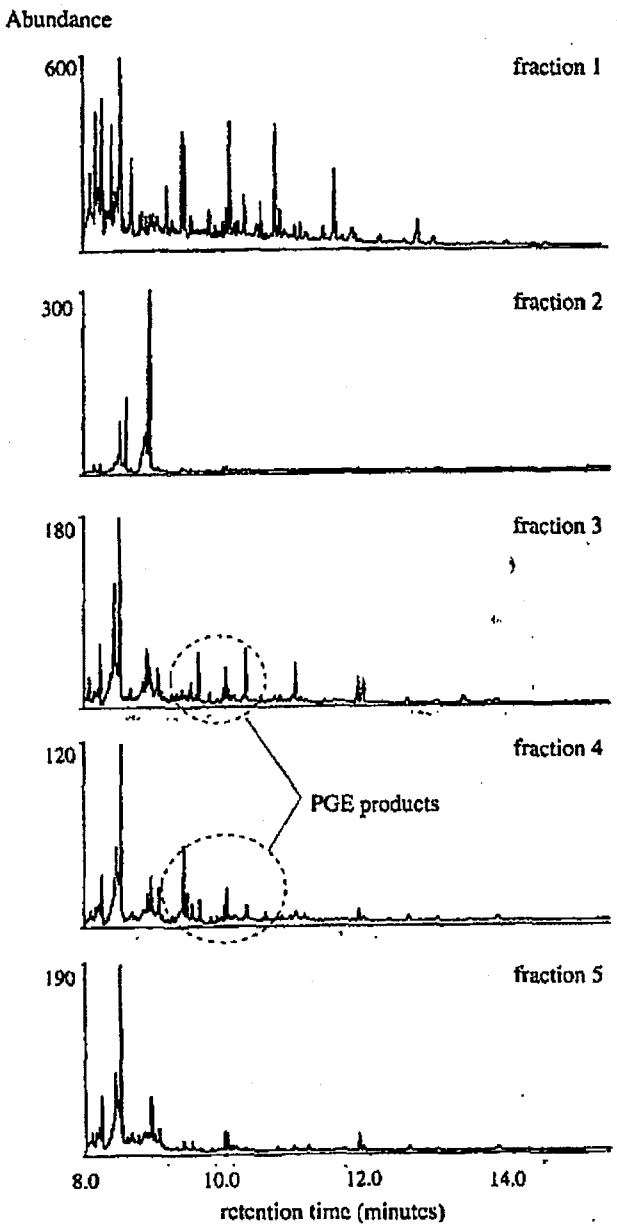

Fig. 7. GCMS analysis of metabolites produced from the enzyme reaction of COX-1 on arachidonic acid.

and DHA (21%) (Murphy et al., 2003). These PUFA are obtained through the food chain, in particular from phytoplankton which are also high in ω3 PUFA (Budge et al., 2001). Sterol analysis shows cholesterol to be the predominant sterol in the mussel, and there is also a variety of other sterols present, including phytosterols (Murphy et al., 2002). Hallam Cove *P. canaliculus* exhibit no significant difference in PUFA or sterol composition when compared to Hallam Cove *M. edulis*, however *P. canaliculus* has a significantly higher total lipid content on a dry weight basis (7.67% compared to 6.00% for *M. edulis*) (Murphy et al., 2003). The lipid content of marine molluscs reportedly varies with season, life cycle and habitat; and biochemical changes may also result from variability in metabolic activity, location, sex and spawning (Kluytmans et al., 1985; Lubet et al., 1985; Marsden and Weatherhead, 1998; Okumus and Stirling, 1998; Hawkins et al., 1999; James et al., 2001; Freites et al., 2002). The mussel species tested here are sampled from the same location at the same time, therefore it is possible that the comparatively higher lipid content of *P. canaliculus* is species specific, rather than related to environmental factors. Apart from the higher lipid composition in *P. canaliculus*, there are no overall significant differences between the two mussel species in lipid class composition. Triglycerides, sterols and phospholipids are the main component lipids, and there is modest free fatty acid content (Murphy et al., 2002).

The complex mixture of long chain PUFA of *P. canaliculus* is thought to contribute to its anti-inflammatory activity. These PUFA are enriched 20 fold in the Lyprinol extract of the dried

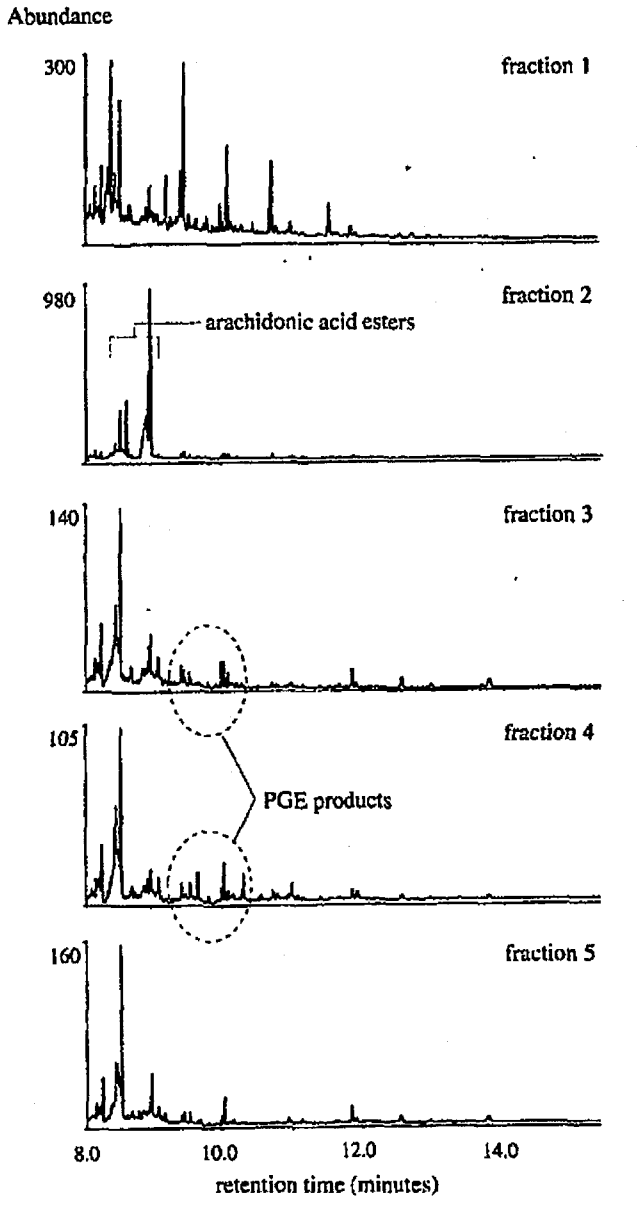

Fig. 8. GCMS analysis of metabolites produced from the enzyme reaction of COX-2 on arachidonic acid.

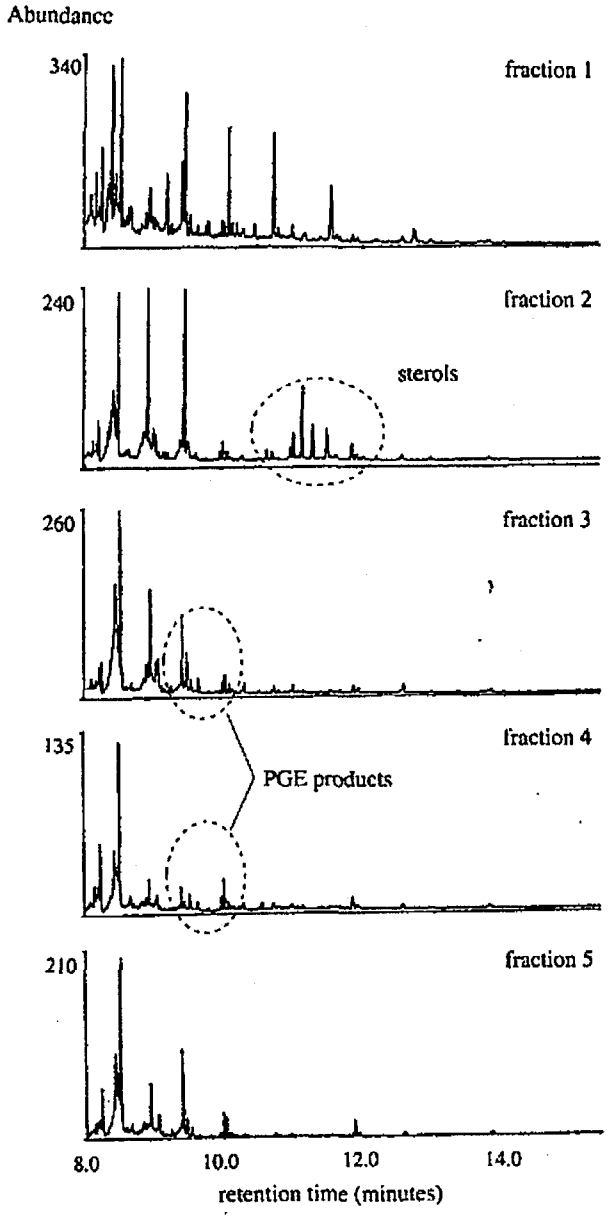

Fig. 9. GCMS analysis of metabolites produced from the enzyme reaction of COX-1 on Lyprinol.

mussel, which is a direct result of the supercritical fluid extraction procedure used to extract the lipid (Whitehouse et al., 1997). Free fatty acids (54%) and triglycerides (27%) constitute the predominant lipid classes of Lyprinol. This composition makes it a very different lipid extract compared to Blackmores fish oil, which is composed mainly of triglyceride (30%) (www.blackmores.com.au). Further investigation of Lyprinol by silver-ion TLC and GCMS reveals a unique mixture of PUFA including EPA (20:5ω-3), DHA (22:6 ω-3), SA (18:4 ω-3), ETA (20:3), DTA (22:3 ω-3), α-LNA(18:3 ω-3) and the rare ω3-AA (20:4 ω-3). In comparison, Blackmores fish oil, which is sourced from oily cold water fish and which may contain anchovy, sardines, salmon and mackerel, is standardised to contain EPA (18%) and DHA (12%) in triglyceride form.

The COX inhibition assay used in this study is based on the oxidation of 1-DCF by the hydroperoxide formed in the cyclooxygenase reaction. The method measures the second peroxidase reaction of the enzyme which has a higher turnover than the initial oxygenase reaction; the oxygenase reaction is considered the limiting step in the overall rate of the enzyme (Larsen et al., 1996). The *P. canaliculus* and *M. edulis* total lipid extracts examined here exhibited little COX inhibitory activity until saponified. The greater inhibition of COX upon saponification has indicated that the free fatty acid fraction is likely to be in part responsible for the anti-COX activity. ω3 Polyunsaturated fatty acids are known to competitively inhibit the COX enzyme (Larsen et al., 1996), and it is likely

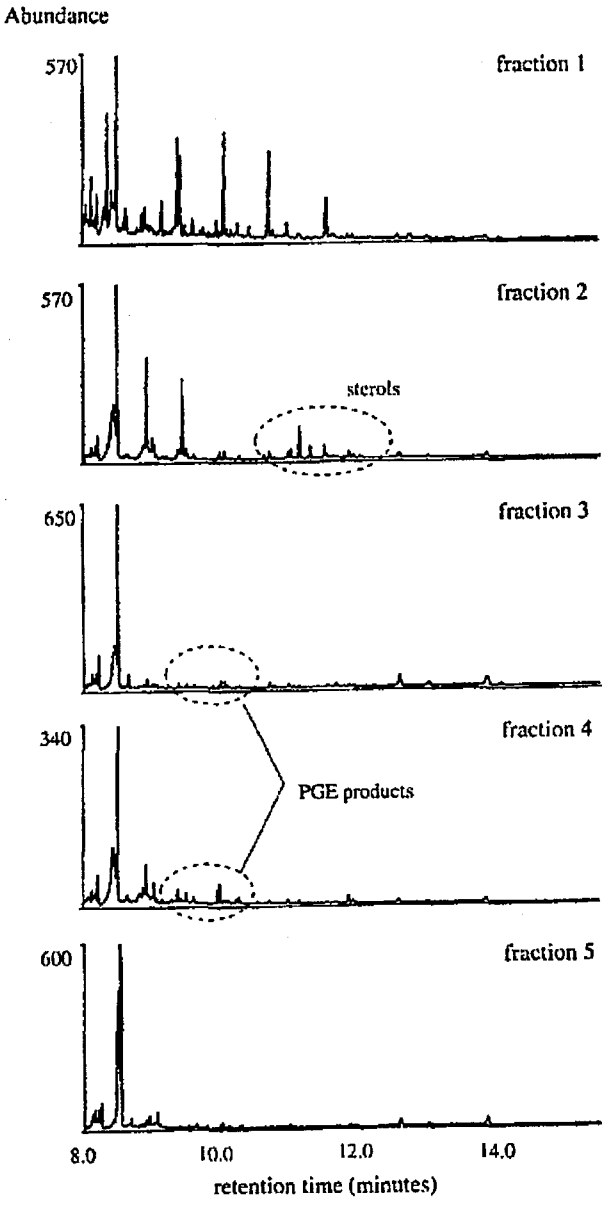

Fig. 10. GCMS analysis of metabolites produced from the enzyme reaction of COX-2 on Lyprinol.

that these PUFA contributed to the anti-COX activity of the saponified fractions.

Lyprinol and hydrolysed Lyprinol exhibited strong inhibition of both COX-1 and COX-2, and did not appear to exhibit selectivity in their inhibition of the two enzymes. There was little difference in inhibitory activity between Lyprinol and hydrolysed Lyprinol when tested at higher concentrations (100 and 1 μg/mL), however, when examining IC_{50} values, hydrolysed Lyprinol has a 10-fold lower IC_{50} value than for Lyprinol. The high percentage of free fatty acids in the Lyprinol extracts supports the argument that the free fatty acid fraction is exerting the inhibitory effect. The enzyme-treated protease and protease–lipase *P. canaliculus* lipid extracts exhibit a similar level of inhibition compared to Lyprinol and hydrolysed Lyprinol at the same concentration, suggesting they contain similar levels of bioactive compounds. Again, it is likely to be the PUFA component that is biologically active; as this conclusion is further indicated by the stronger inhibition exhibited by the PL extract compared to the P extract. The enzyme-assisted solvent extraction of *P. canaliculus* mussel meat utilises a proteolytic enzyme to facilitate oil release, and a lipase enzyme to increase the yield of the highly bioactive free fatty acid fraction of *P. canaliculus*.

Fish oil at both 100 μg/mL and 1 μg/mL exhibited less COX inhibitory activity compared to Lyprinol at the same concentrations. Upon hydrolysis, fish oil exhibited similar inhibition at 1 μg/mL to Lyprinol and hydrolysed Lyprinol. Fish oil is predominantly composed of triglyceride molecules which are rich in EPA and DHA, while Lyprinol is a more complex mixture of lipid classes and is particularly high in free fatty acids. Seemingly, hydrolysis of fish oil results in the release of inhibitory PUFA giving it a similar activity to Lyprinol.

There are at least two isoforms of mammalian COX, the constitutive COX-1 enzyme, and COX-2 which is induced by inflammatory stimuli. Although inhibition of COX-2 is beneficial to the reduction of the inflammatory response, there is debate concerning the safe usage of NSAIDs and selective COX-2 inhibitors as anti-inflammatory agents in relation to gastrointestinal and cardiovascular events (Parente and Perretti, 2003). In the present study, Lyprinol and the lipid extracts from *P. canaliculus* exhibited no inhibition selectivity or preference for either COX-1 or COX-2, and this was apparent for the extracts at various concentrations. This observation is in contrast to the NSAID indomethacin, which is a non-selective inhibitor of COX that shows preferential activity against COX-1 (Hull et al., 2003). At comparable concentrations, Lyprinol is as effective as Indomethacin at inhibition of COX-1, but is much more effective at inhibition of COX-2.

Upon separating Lyprinol into its constituent lipid classes, the free fatty acid fraction exhibited the highest inhibition of the Lyprinol lipid classes, and it is likely that the unique mixture of ω-3 PUFA in Lyprinol, including EPA and DHA contained within this fraction, act directly as competitive substrate inhibitors of AA metabolism by COX. The triglyceride fraction of Lyprinol also exhibited some inhibitory activity, but the mechanism of action for this is unclear. In most marine organisms the majority of fatty acids occur predominantly as phospholipids (Volkman et al., 1998), and then as triglycerides. Triglycerides are energy storage lipids and can be used as a condition index for marine organisms (Parrish et al., 2000).

Sterol compounds can be highly bioactive, and various sterols have been known to influence human health and disease, the most cited example being cholesterol which has a harmful effect on cardiovascular health (Patel and Thompson, 2006). Cholesterol is identified as the major sterol in both *P. canaliculus* and *M. edulis* (Murphy et al., 2003), and this has some implications when considering the mussel preparations as a dietary treatment. Altogether, 18 individual sterols are identified in *P. canaliculus* and *M. edulis*, with desmosterol/brassicasterol (coeluting), campesterol, *trans*-22-dehydrocholesterol and 24-nordehydrocholesterol accounting for over 65% of the remaining sterol fraction (Murphy et al., 2002). Phytosterols, such as β-sitosterol which is also reported in *P. canaliculus*, are known to generate anti-inflammatory activity in acute animal inflammation models (Li and Sinclair, 2002). In this study, the sterol and sterol ester fractions exhibited no significant inhibition of COX-1, although there was some inhibition of COX-2. The reason for this selectivity is unclear. It is possible that certain sterol compounds, including phytosterols derived from the mussel's phytoplankton diet, exhibit a positive, albeit small, effect on COX-2 inhibition.

The phospholipids (polar lipid) fraction of Lyprinol also exhibited moderate inhibition of COX-2, while COX-1 inhibition was not significant. Phospholipid molecules can be highly bioactive and may contribute to the COX-2 inhibition exhibited (Eligini et al., 2002). Furthermore, there could be a variety of other polar compounds (oxidised PUFA, glycolipids, partially hydrolysed lysophospholipids and lysoglycolipids) in the fraction that may be responsible for the anti-COX activity seen.

In relation to the purified PUFA tested in this study, SA, ω3-AA, α-LNA and EPA all exhibited high inhibition of both COX isoforms. Certain PUFA are known to inhibit COX by acting as competitive substrate inhibitors. For example, EPA is known to be a substrate, albeit a poorer one than AA, for prostaglandin biosynthesis (Corey et al., 1983). Also, research has shown that prostaglandins of the ω3-series, derived from EPA, are less biologically active, and ω3-AA has been shown to be as potent as EPA in inhibiting COX (Croset et al., 1999). Although not as active as the above mentioned PUFA, the 22 carbon length fatty acids, AdA, DHA, and DPA exhibited high inhibition of the COX isoforms in this study. Of interest, DHA and DPA exhibited higher inhibition of COX-2 compared to COX-1. Docosahexaenoic acid is not an alternate substrate for mammalian COX but it is reported to be a potent competitive inhibitor of the enzyme (Corey et al., 1983). Also, DPA is shown to inhibit or influence the activity of COX in various experiment models (Akiba et al., 2000). Eicosatrienoic acid and DTA, meanwhile, only exhibit moderate inhibition of COX-1, although inhibition of COX-2 is still strong. These results indicate that the three bond configuration of these PUFA is not greatly effective at inhibiting COX-1, although they are still highly active in COX-2 inhibition, and this is likely to be due to the larger COX-2 active site that can accommodate PUFA

substrates more readily (Vane et al., 1998). Significant inhibitory effect is also shown by ω6-AA, when pre-incubated for 5 min with both COX-1 and COX-2. This observed inhibition may be attributed to a possible autocatalysed self-inactivation of the COX enzyme in the presence of excess arachidonic acid substrate (Larsen et al., 1996). It is also possible that the inhibition observed by the various bioactive PUFA occurs via a similar mechanism.

The present investigation of prostaglandin metabolite production by COX employed an *in vitro* enzyme system, which aimed to simplify the investigation of the Lyprinol interaction with COX by eliminating further metabolism by other enzymes in the arachidonic cascade, which would otherwise occur in a whole cell or *in vivo* assay. The pure enzyme reaction studies, as discussed above, have shown Lyprinol to inhibit the turnover reaction of AA by the COX isoforms. However, when Lyprinol without the presence of AA is incubated with COX, the GCMS results also indicate the presence of prostaglandin metabolites, suggesting that alternate PUFA substrates from Lyprinol are being converted to prostaglandin analogues. This designates the mechanism of inhibition by Lyprinol as that of a competitive substrate inhibitor. Lyprinol, like other marine oils, is high in ω3 PUFA such as EPA and DHA. Studies have shown EPA-derived prostaglandin metabolites to be produced via the COX reaction, and these metabolites are considered to be less biologically active than their AA counterparts (Corey et al., 1983). Docosahexaenoic is also reported to be a substrate for COX (Larsen et al., 1996). It follows that other Lyprinol PUFA could be metabolised to alternate prostaglandin metabolites with unknown biological actions in different cell types. For example, ω3-AA is not known to be converted into prostaglandins by COX, however platelet COX is shown to produce 12-hydroxy-8,11,14-heptadecatrienoic acid from this PUFA (Croset et al., 1999). For these Lyprinol reactions, it is noted that the prostaglandin metabolite peaks are relatively abundant indicating that alternate PUFA substrate is not metabolised at a slower rate than AA. This is in contrast to literature which reports that the PUFA substrates, α-LNA, AdA and EPA, are substrates for COX with decreasing reaction rates in the mentioned order, behind AA (Larsen et al., 1996). However the long incubation time of 15 min for each reaction may result in a defined metabolite concentration before auto-catalysed inactivation of the COX enzyme occurs.

There has been debate regarding the bioactive components of *P. canaliculus*. Many researchers believe it to reside in the lipid component of the mussel (Whitehouse et al., 1997; Gibson and Gibson, 1998), while other groups have reported the protein (Couch et al., 1982; Mani et al., 1998) and glycogen (Miller et al., 1993; Tan and Berridge, 2000) fractions to be efficacious. Of importance in these clinical trials is the fact that Seatone and Lyprinol have shown to be well tolerated, with virtually no adverse side effects (Rainsford and Whitehouse, 1980; Whitehouse et al., 1997; Shiels and Whitehouse, 2000), and this is in contrast to traditional anti-inflammatory treatments such as NSAIDs and the more recent COX-2 specific inhibitors, which can have adverse effects especially when taken for long periods of time. Lyprinol and particular fractions from *P. canaliculus* that are high in PUFA are shown in this study to exhibit strong inhibition of the COX isoenzymes, and are postulated to do so by acting as alternate substrates for these enzymes. These results support the use of the commercial mussel extracts, in particular Lyprinol, as an alternative for conventional NSAIDs and fish oil treatment in the relief of the symptoms of arthritis.

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Monday, 30 October 2000

Dear Sir / Madam

Re.- Comparison of Lyprinol with Fish Oils

John Waitzer has asked me to comment on the comparative effectiveness of fish oils and Lyprinol in the treatment of inflammatory conditions.

One of the main differences between Lyprinol and oils is the amounts required to produce significant clinical effects. In many of the Clinical Trials on the effectiveness of fish oils (including those of Lee, TK et al and Cleland LG et al) the doses of fish oil used were as high as 18 grams/day (18 x 1 gm capsules). This is a very large number of capsules, and represents some 18 grams of fat. In the case of Lyprinol, significant clinical effects are seen at a maintenance does of 2 capsules/day (equivalent to 100 milligrams of Lyprinol oil and 300 mg of olive oil - a total of 400 mg of fat).

This also introduces the cost benefit of Lyprinol. At these doses, the cost of Lyprinol of approximately 50 cents US/day, which should compare very favourably with fish oils at 18 capsules/day. The trouble is that most people using fish oils are not taking it at these high doses (probably less than 4 /day) and hence are not receiving an appropriate benefit.

While there have been no direct clinical human studies comparing the effectiveness of Lyprinol with fish oils, studies have been performed in rats using the adjuvant model of arthritis. These studies have been performed by Dr Michael Whitehouse, and I believe these publications have been drawn to your attention. These studies formed the basis of the claims that Lyprinol is 200 times more potent than Max EPA (fish oil), etc.

The putative mode of action of both Lyprinol and fish oils are on the metabolism of arachidonic acid, with the EPA (eicosapentaenoic acid) component of the fish oil competing with arachidonic acid (AA) fractions in cell membranes for metabolism via the cyclo-oxygenase and lipoxygenase pathways. EPA is a competitive substrate for arachidonic acid, which means that its inhibitory effect is via mass action. That is, the more EPA in your cells the better it competes with endogenous membrane arachidonic acid. The EPA is metabolised into leukotrienes of the 5 series (e.g. LTB_5) and prostaglandins of the 3 series (e.g., PGB_3), whereas AA is metabolised into 4 series leukotrienes (e.g., LTB_4), a series 2 prostaglandins (e.g., PGB_2). These series 5 leukotrienes and series 3 prostaglandins still retain some biological activity. This is why such large doses of fish oils are required to produce significant clinical effects, and why the effects are generally slow in onset (greater than 4 weeks) However, Lyprinol is not metabolised at all, but directly inhibits the 5-lipoxygenase and cyclo-oxygenase enzymes, the first steps in the lipoxygenase and cyclo-oxygenase

pathways respectively. Whether or not this inhibition is via permanent binding to these enzymes has not been determined yet. This explains the more potent effects of Lyprinol, and why it acts more like a standard non-steroidal anti-inflammatory drug, and why it exerts its clinical effects more quickly (e.g. less than 12 days).

I hope these comments - a little technical I know - have been of some assistance. Please don't hesitate to contact me if you require any additional information or if you wish to discuss any of the above issues in more detail.

Yours sincerely,

Henry Betts, PhD