

ANTI-INFLAMMATORY ACTIVITY OF A LIPID FRACTION (LYPRINOL) FROM THE NZ GREEN-LIPPED MUSSEL

M.W. WHITEHOUSE^{1*}, T.A. MACRIDES², N. KALAFATIS², W.H. BETTS³,
D.R. HAYNES⁴ AND J. BROADBENT⁵

¹Department of Medicine, University of Queensland, Princess Alexandra Hospital, Brisbane, Queensland 4102; ²Department of Medical Laboratory Science, Royal Melbourne Institute of Technology, Victoria 3000; ³Rheumatology Unit, The Queen Elizabeth Hospital, Woodville, South Australia 5011; ⁴Department of Pathology, University of Adelaide, South Australia 5005; ⁵McFarlane Laboratories, Surrey Hills, Victoria 3127, Australia

*Correspondence

ABSTRACT

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A lipid-rich extract, prepared by supercritical fluid extraction of fresh stabilized mussel powder (Lyprinol), showed significant anti-inflammatory (AI) activity given therapeutically and prophylactically *po* to Wistar and Dark Agouti rats developing either (a) adjuvant-induced polyarthritis or (b) collagen(II)-induced autoallergic arthritis, with $ED_{50} \leq 15$ mg/kg, c.f. naproxen ≥ 25 mg/kg or various therapeutic oils (flaxseed, evening primrose, fish) ≥ 1800 mg/kg given orally. Lyprinol showed little or no activity in acute irritation assays (carrageenan, kaolin, histamine) indicating it is not mimicking rapid-acting NSAIDs.

Incorporating Lyprinol into arthritogenic adjuvants composed of heat-killed *Mycobacterium tuberculosis* suspended in olive oil or squalane, effectively prevented arthritis development at a dose of 5 mg/rat. By contrast, 'dummy adjuvants' prepared with *Mycobacterium tuberculosis* and flaxseed, evening primrose or fish oils were still arthritogenic in Dark Agouti rats (doses of oil = 90 mg/rat).

Lyprinol subfractions inhibited leukotriene- B_4 biosynthesis by stimulated human polymorphonuclear leukocytes *in vitro*, and prostaglandin- E_2 production by activated human macrophages *in vitro*. Much of this AI activity was associated with polyunsaturated fatty acids and natural antioxidants (carotenoids, etc.).

In contrast to NSAIDs, Lyprinol is non-gastrotoxic in disease-stressed rats at 300 mg/kg *po* and does not seem to affect platelet aggregation (human, rat). These data show Lyprinol to be a reproducible, relatively stable, source of bioactive lipids with much greater potency than plant/marine oils currently used as nutritional supplements to ameliorate signs of inflammation.

Keywords: NZ green-lipped mussel, Lyprinol, lipid fraction, inflammation, NSAIDs

INTRODUCTION

There is a tradition of using shellfish supplements as a remedy for arthritis among indigenous people, notably in Western Mexico and throughout the South Pacific. The dried flesh of the NZ mussel *Perna canaliculus*, has been sold in several countries since the early 1970s as a nutritional supplement (Seatone) with possible benefits for

relieving arthritis [1,2]. Reports of early clinical trials have been mixed: some attesting benefits [3–6], others not [7–10]. Studies using animals have likewise yielded mixed findings. Acute anti-inflammatory activity in rats was associated with protein [11] or glycogen fractions [12] but only when injected ip. The product available in the 1970s showed modest or no activity in suppressing the carrageenan paw oedema [13] and had no effect on the adjuvant-induced arthritis in rats [14] when given orally. Indirect evidence was adduced for the presence of an anti-histamine (perhaps lysolecithin [15]) and anti-prostaglandin activity, as indicated by delayed parturition in rats [16].

The situation changed dramatically in 1986 when dried mussel extracts became available that had been stabilized with 3% tartaric acid immediately after removing the flesh from the shell [17]. This additive acts as both a metal chelator and antioxidant, in effect preserving labile unsaturated molecules from spontaneous (possibly metal-catalyzed) auto-oxidative destruction.

Lyprinol is the mussel 'oil', obtained by supercritical fluid extraction (SFE) of this stabilized mussel powder using liquified carbon dioxide [18]. It represents about 4–5% original dry weight and contains triglycerides, sterol esters, free fatty acids, polar lipids and carotenoids but no solvent residues. As this report shows, the bulk of the anti-inflammatory activity of stabilized Seatone is extracted into this lipid fraction so that Lyprinol effectively constitutes a 20-fold concentrate of the original dried mussel. Being very low in protein it is unlikely to be allergenic and being salt-free it is more acceptable for cardiac patients than the original dried mussel preparations.

METHODS

Materials

Stabilized Seatone (McFarlane Laboratories, Surrey Hills, Victoria, Australia) was purchased from a local pharmacy and kept at room temperature. The SFE extract, Lyprinol capsules (containing 50 mg of this extract with 200 mg olive oil) and the bulk residue of the mussel powder remaining after SFE extraction were all supplied by McFarlane Laboratories and kept at 4°C. Other oils used as nutritional supplements were purchased from health food stores (local and overseas). The preparation of Lyprinol subfractions is described elsewhere [19].

Assessment of anti-inflammatory activity

This was assessed in rats after oral administration of either (a) aqueous dispersions prepared with 0.2% Tween-20 as a suspending agent or (b) unemulsified lipids, diluted into cold-pressed olive oil as necessary. Acute rear paw inflammation was induced with 0.6 mg carrageenan in 0.1 ml saline injected into both rear paws of female Wistar rats. Chronic polyarthritis was induced with adjuvants prepared from dried *Mycobacterium tuberculosis* (0.3–1 mg) dispersed in 0.1 ml squalane olive oil or other oily vehicles, and inoculated into the tailbase of Wistar or Dark Agouti rats on day 0. Treatment was

given either from day minus -1 continually for 16 days (i.e. a prophylactic regime) or from day 10 through 13 (therapeutic regime) i.e. after the first signs of arthritis were manifest. Rear paw inflammation was measured with a micrometer. Forepaw inflammation was assessed arbitrarily on a scale of 0-4+. An independent observer assigned an overall arthritis score to all animals based on paw/tail inflammation and overall condition/mobility.

A second prophylactic assay was based on treating female Dark Agouti rats (150-180 g) with test fractions/oils co-administered with the mycobacterial arthritigen.

An autoallergic arthritis was induced in Wistar rats [20] by inoculating into the right rear paw 200 µg bovine cartilage collagen Type-II emulsified with a complete Freund's adjuvant (Difco Laboratories, Detroit, USA). (This commercial adjuvant is too weak to induce adjuvant disease in this rat strain.) These animals were treated orally from day 8 onwards.

In-vitro studies

Leukotriene biosynthesis by human polymorphonuclear leukocytes (PMN) was studied as described [21]. After isolation, 1 ml of PMN was added to glass tubes and warmed to 37°C for 5 min before the addition of 5 µl of test fractions or 5 µl methanol (control). After 10 min the PMN were stimulated to produce leukotrienes by the addition of arachidonic acid (10 µmol/L final volume) and the calcium ionophore, A23187 (5 µmol/L final volume). After a further 5 min incubation, leukotriene synthesis was stopped by the addition of 100 µl of 100 mmol/L citric acid and LTB₄ and 5-HETE measured by HPLC [21].

Prostaglandin production by human monocytes was measured as follows. Mononuclear cells were isolated using a ficol gradient [22] and 4×10^6 cells were suspended in 250 µl of RPMI-1640 medium supplemented with 10% fetal calf serum, 5 µg/ml penicillin and 50 U/ml streptomycin. After incubation at 37°C in 5% CO₂ for 1 h, the non-adherent cells were removed by washing with Hanks balanced salt solution. The adherent cells were then incubated in 250 µl of the RPMI medium containing the fatty acid methyl esters prepared from Lyprinol subfractions at various concentrations and 5 µg/ml *E. coli* lipopolysaccharide. After 24 h the supernatants were sampled and analysed for prostaglandin-E₂ using a competitive radioimmunoassay [23].

Platelet aggregation was measured *ex vivo* using 400 µl platelet-rich plasma, obtained from rats by cardiac puncture using heparin or from volunteers by venipuncture using citrate as anticoagulant. Aggregation at 37°C was triggered by adding 10 µl 25 mmol/L arachidonic acid or 10 µl 0.2 mmol/L ADP and recorded using a Chronolog aggregometer. Wistar rats were pre-dosed orally for up to 16 days with 50 mg kg⁻¹ day⁻¹ Lyprinol. Volunteers took either 150 mg aspirin or 3 Lyprinol capsules (each containing 50 mg) per day for 7 days before drawing the blood samples.

RESULTS

Inhibition of adjuvant-induced polyarthritis in Wistar rats

Table 1 compares the efficacy of the original stabilized mussel powder (Seatone) with the two derived fractions; namely, the SFE extract, which constitutes approximately 4% and the residues being the residual $\geq 95\%$ of the extracted dried mussel. The top dose of 300 mg/kg was the same as that required for aspirin to show any effect on this experimental arthritis. Naproxen and ibuprofen were included in this comparison, being readily available in over-the-counter (OTC) formulations in Australia and other countries. All the test drugs/materials were given orally for the maximum (prophylactic) dosing period = 16 daily doses beginning on the day before inoculating the arthritigen.

Table 2 shows the results of dosing according to this maxi(prophylactic) schedule with the SFE extract in olive oil (Lyprinol) and a range of plant/marine oils currently sold OTC with implied claims that they may confer benefit in inflammatory disorders. None of these plant/marine oils matched Lyprinol in potency.

Table 3 also compares Lyprinol with these plant/marine oils in a much briefer therapeutic or dosing schedule: namely treating the animals from the time of initial onset of arthritis (day 10 post-arthritigen) for only 4 days. Following cessation of this brief treatment (on day 13), Lyprinol-treated rats exhibited a slow recrudescence of arthritic signs from day 14 onwards, indicating that the therapeutic effect is reversible though the rebound was noticeably slower than post-NSAID treatment. The plant/marine oils were barely effective in this short-term dosing experiment.

TABLE 1
Adjuvant arthritis – prophylactic treatment with mussel preparations or (OTC) NSAIDs

Treatment	Dose mg/kg	Signs of arthritis (\pm SE) on day 15		
		Rear paw swelling (mm)	Front paw swelling (mm)	Arthritis score
None	–	1.19 (0.21)	2.5 (0.3)+	2+
Dried mussel	300	0.24 (0.05)	0.8 (0.2)+	0.7+
SFE ex mussel	15	0.23 (0.06)	1.3 (0.2)+	0.5+
Mussel residue	300	1.15 (0.09)	1.9 (0.7)+	2+
Aspirin	300	0.72 (0.16)	1.9 (0.4)+	1.5+
Ibuprofen	50	0.61 (0.02)	1.7 (0.1)+	1.4
Naproxen	25	0.25 (0.17)	0.8 (0.3)+	0.8+

Test materials administered po for 16 days, female Wistar rats ($n = 4/\text{group}$)

TABLE 2
Adjuvant arthritis: prophylactic treatment with Lyprinol or some plant/marine oils

Treatment*	Dose mg/kg	Signs of arthritis (\pm SE) on day 15		
		Rear paw swelling (mm)	Front paw swelling (mm)	Arthritis score
None	–	1.21 (0.07)	2.8 (0.5)+	2.8+
Olive oil	1850	1.19 (0.12)	2.9 (0.3)+	2.7+
Lyprinol	20	0.23 (0.13)	1.4 (0.3)+	1.5+
Fish oils				
Max EPA	1850	0.63 (0.22)	1.1 (0.3)+	1.8+
Norwegian salmon	1850	0.74 (0.11)	2.7 (0.8)+	2.0+
Pikasol	1850	1.36 (0.11)	3.0 (0.3)+	3+
Plant oils				
Flaxseed	1900	1.07 (0.22)	3.3 (1.1)+	1.7+
Evening primrose	1900	0.82 (0.22)	2.5 (0.9)+	1.5+

Oils administered po for 16 days, female Wistar rats ($n = 5$ /group)

*Source of oils: Max EPA (Solgar, Lynbrook, NY, USA); Norwegian salmon (J.R. Carlson, Arlington Hts, IL, USA); Pikasol (Lube AS, Hadsund, Denmark); Flaxseed (Barleans, Ferndale, WA, USA); Evening primrose (Nature's Way, Springville, Utah, USA)

Other studies of arthritis inhibition

Table 4 shows that Lyprinol also suppresses the autoallergic arthritis induced by sensitizing Wistar rats to the unique collagen Type-II present in cartilage. Fish oils may actually augment this auto-allergic arthritis in rats [24]. Ibuprofen had no effect on the acute/chronic paw swelling induced in the right rear paw by the injection of the antigen/adjuvant emulsion. Lyprinol did reduce this local inflammation as well as the systemic arthritic manifestations in the other paws.

Table 5 shows that incorporating the SFE from the mussel into the arthritogenic adjuvants, effectively ablated arthritis development. This is a rare property of only a few drugs currently used to treat arthritis, notably cyclosporin and lobenzarit [25]. A pseudoadjuvant prepared from the contents of the Lyprinol capsule (i.e. the SFE in olive oil) was not arthritogenic in contrast to plain olive oil adjuvants.

TABLE 3
Adjuvant arthritis: therapeutic treatment with Lyprinol or some plant/marine oils

Treatment*	Dose mg/kg	Mean changes in signs of arthritis (\pm SE)		
		Rear paw swelling (mm)	Front paw swelling (mm)	Arthritis score
None	–	1.09 (0.27)	1.8 (0.5)+	2.6+
Olive oil	1850	0.96 (0.11)	1.9 (0.2)+	2.7+
Lyprinol	20	0.02 (0.10)	+ (0.5)	1.5+
Fish oils				
Max EPA	1850	0.91 (0.23)	2.1 (0.4)+	2.1+
Norwegian salmon	1850	1.38 (0.43)	2.6 (1.0)+	2.8+
Plant oils				
Flaxseed	1900	0.75 (0.24)	1.6 (0.6)+	1.8+
Evening primrose	1900	0.69 (0.17)	1.8 (0.5)+	1.8+
Ibuprofen	40	0.80 (0.14)	1.9 (0.2)+	2.3+

Test materials administered po for 4 days only, female Wistar rats ($n = 5$ /group)

*Source of oils: see Table 2

In-vitro studies

These were conducted with various subfractions prepared from the original SFE extract [18,19]. Free fatty acids (constituting about 30% of the SFE extract) were separated by reverse-phase chromatography. Fractions from the column were collected at various time intervals.

Table 6 shows that four of these fractions (f,d,b,e) were particularly effective in inhibiting the transformation of added arachidonate to leukotriene-B₄ and 5-HETE by A-23187-activated human PMN. These active fractions contain polyunsaturated acids with 4, 5 and 6 double bonds.

The unfractionated SFE material also inhibited prostaglandin E₂ production from endogenous arachidonate by stimulated human blood monocytes with IC₅₀ = 1.2 µg/ml. This latter finding confirms a previous prescient observation that a mussel preparation mimics NSAIDs in prolonging gestation in rats by influencing prostaglandin production [16].

TABLE 4
Collagen-II arthritis: therapeutic treatment with mussel preparations

Treatment	Dose mg/kg	Signs of arthritis on day 15 (\pm SE)		
		Left rear paw swelling (mm)	Right rear paw swelling (mm)	Forepaw inflammation
None	—	1.77 (0.17)	1.62 (0.27)	2.5 (0.5)+
Olive oil	1850	1.75 (0.05)	1.75 (0.35)	2.3 (0.6)
SFE in olive oil ^a	20	0.32 (0.07)	1.04 (0.04)	0.6 (0.1)+
Whole mussel ^b	300	0.74 (0.16)	1.11 (0.16)	0.8 (0.2)+
OTC ibuprofen ^c	50	0.82 (0.19)	1.48 (0.39)	0.7 (0.2)+

Female Wistar rats sensitized by injecting C-II/CFA in right rear paw (day 0). Treatment given po from day 8 onwards ($n = 4$ animals/group)

^aAs in Lyprinol = 20% SFE in olive oil; ^bSeatone (Australia); ^cNurofen

TABLE 5
Arthritis ablation by mussel 'oil'/Lyprinol

<i>Mycobacterium tuberculosis</i> in	SFE (mg/rat)	Signs of arthritis (\pm SE)				Δ wt (g)
		Rear paw swelling (mm)	Front paw swelling (mm)	Arthritis score		
Olive oil	—	1.68 (0.31)	4 (0.7)+	3.2+	+04	
	10	0.19 (0.22)	0.3 (0.2)+	0.2+	+20	
	5	0.46 (0.26)	1.2 (0.3)+	1.5+	+17	
	2	0.88 (0.61)	1.3 (0.7)+	2+	+18	
Lyprinol ^a	(20)	0.13 (0.17)	0.8 (0.3)+	0.2+	+25	
Flaxseed oil	—	1.47 (0.27)	3.8 (0.9)+	3+	+05	
Tri-GLA ^b	—	1.08 (0.43)	3.5 (1.4)+	2.3+	+11	
Pikasol ^c	—	0.78 (0.15)	1.3 (0.2)+	1.1+	+16	

Female Dark Agouti rats ($n = 4$ /group) inoculated with 0.1 ml mycobacterial/oil 'adjuvants' containing mussel lipids (SFE). Ensuing arthritis scored on day 17

^aLyprinol = 20% SFE in olive oil; ^bTri- γ -linolein (80%, Scotia); ^cDanish fish oil

TABLE 6
Inhibition of human PMN leukotriene production by Lyprinol and its subfractions

Experimental conditions	Fatty acid concentrations (µg/ml)	Retention time (min)	LTB ₄ ng/10 ⁶ PMN (±SE)	5-HETE
Control PMN			19.8 ± 0.8	129 ± 3
Unfractionated SFE	100		0	12 ± 7
SFE fractions				
RPFA-a	9	18–22	19.7 ± 1.6	156 ± 16
RPFA-f	41	22–25	0	0
RPFA-d	30	25–31	4.2 ± 0.8	0
RPFA-b	46	31–36	0	0
RPFA-e	18	36–42	2.2 ± 1.1	24 ± 4
RPFA-i	20	42–47	16.7 ± 1.2	95 ± 6
RPFA-c	113	47–61	15.7 ± 1.1	110 ± 4
RPFA-g	155	61–66	17.0 ± 0.6	103 ± 4
RPFA-h	51	77–83	16.8 ± 0.9	134 ± 4

Other in-vitro studies

Lyprinol, like Seatone, showed little activity in the standard test for NSAIDs, namely rapid inhibition of the carrageenan paw oedema (data not shown). Lyprinol was non-gastrotoxic in fasted disease-stressed (arthritic) rats at a dose of 300 mg/kg. The same dose of aspirin caused massive gastric haemorrhage.

Preliminary studies indicated that platelet aggregation *ex vivo*, triggered by arachidonate or ADP, was normal in platelet-rich plasma from rats dosed with Lyprinol for 16 days (at 50 mg/kg) and two human volunteers taking 150 mg/day Lyprinol. Aspirin at the same daily dose profoundly inhibited platelet aggregation induced by arachidonate but had little/no effect on aggregation induced by ADP.

DISCUSSION

These in-vivo/in-vitro studies indicate that Lyprinol, the lipid fraction (mussel 'oil') obtained from stabilized dried mussel powders, is a potent but relatively slow-acting anti-inflammatory agent. It behaves as a dual inhibitor of arachidonate oxygenation by both cyclooxygenase (COX) and 5-lipoxygenase. Since Lyprinol does not seem to readily affect the natural resistance of the stomach mucosa or inhibit platelet aggregation, this effect on cyclooxygenase activity may be directed more towards the inducible (inflammation-associated) COX-II rather than the 'housekeeping' COX-I (gastric mucosa, platelet).

Extensive fractionation of Lyprinol yielded more potent but rather less stable subfractions, particularly when these were enriched at the expense of the endogenous antioxidants. The finding of two or three particular unsaturated fatty acids, other than EPA or DHA, is particularly interesting as they appear to be potential antimetabolites to arachidonate [19]. The characterization of these mussel-sourced acids is described elsewhere [26].

Other polyunsaturated fatty acids have been extensively studied as potential immunoregulators [27]. The general consensus is that rather high doses of α - or γ -linolenates, EPA or DHA are required to consistently influence immune responses as determined both in vivo and in vitro. These novel mussel acids, together with the EPA and DHA (and perhaps other unidentified components) also present in Lyprinol, may act synergistically as a potent immunomodulating combination of natural drugs.

These studies certainly give credibility to the folk wisdom of using certain marine products, particularly this New Zealand-sourced mussel, as a nutritional supplement to help alleviate the signs and symptoms of arthritis.

The leukotriene-regulant activity may also be of interest in other clinical contexts (e.g. asthma, inflammatory bowel disease).

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