A NOVEL, POTENT, ORALLY-ACTIVE NATURAL ANTI-ANGIOGENIC PREPARATION

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Abstract

In healthy, mature older adults, angiogenesis (growth of new blood vessels) is virtually non-existent. When it occurs in such individuals, it is frequently associated with a pathological condition. Among these are tumour growth and metastasis, age-related macula degeneration, retinopathy and rheumatoid arthritis. Antagonists are beneficial for treating these chronic conditions.
We have previously shown that shark cartilage is anti-angiogenic when administered orally. Subsequent research has demonstrated that this activity could be ascribed to a lipoid fraction. Lipid-rich extracts were isolated from a number of fish species and shark organs and assayed. The ethanolic extract from shark muscle was particularly potent. This preparation, *macolipin,* which is especially rich in polyunsaturated fatty acids (notably docosahexaenoic acid), produced a 50% inhibition at 5μg/ml. When administered orally macolipin elicited a 50% reduction in angiogenesis at 17mg/kg body weight.
Several plant or vegetable oils including olive oil were shown to have no inhibitory effect in the aortic ring or the *in vivo* models. However, macolipin, when combined with olive oil (1:9) is twice as potent in the *in vitro* system. This combination is 6 to 7 times more active when administered orally to rats.
Preliminary experiments suggest that this combination can negate the stimulatory effects of factors such as VEGF, FGF-2 and TGF-β. This suggests that Macolipin/Olive oil (1:9) mixture is a potent orally-active natural product that has significant anti-angiogenic effects.

Introduction

In healthy adults angiogenesis is mostly associated with diseases and pathological conditions.

Diseases in which angiogenesis is important include:

- CANCER
- MACULA DEGENERATION
- PSORIASIS
- METASTASIS
- RETINOPATHY
- RHEUMATOID ARTHRITIS

Since angiogenesis is minimal in most healthy adults, inhibition of it is unlikely to have adverse or unpleasant side-effects.

Inhibition of angiogenesis is probably beneficial in the treatment of these diseases.

This presentation discusses a natural product that displays potent anti-angiogenic activity.

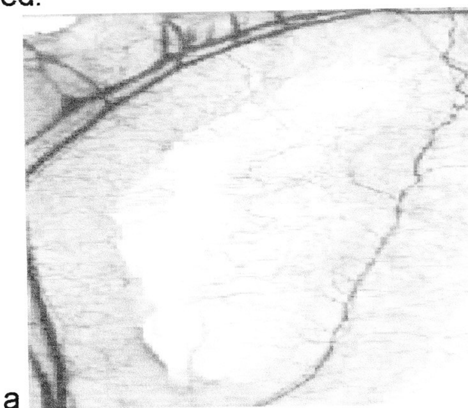
Background

Some years ago shark cartilage was proposed as being beneficial for the treatment of solid cancers because of its purported anti-angiogenic ability.

We showed that it did indeed have anti-angiogenic activity when assayed *in vitro* (rat aortic ring assay).

EVEN MORE IMPORTANTLY, it was anti-angiogenic when administered orally to rats. This indicated that it could inhibit angiogenesis when taken via mouth [Microvasc Res. **54** 178-182 (1997)].

It also showed that the anti-angiogenic component(s) of the shark cartilage are active and so must be able to pass through the digestive system without being degraded.



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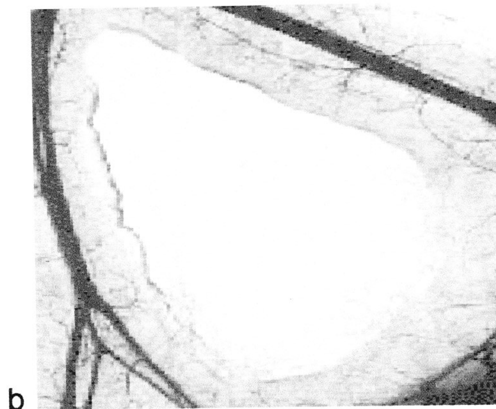

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Anti-Angiogenesis and Shark

Subsequent research showed that a lipid-rich fraction from the shark cartilage was potently active as an anti-angiogenic preparation (Data not shown).

Although shark is a cartilaginous fish the lipid content is not high.

So lipid-rich fractions from other organs of shark were assayed for anti-angiogenic activity.

The most active anti-angiogenically were:

Muscle

Spinal Cord

Anti-Angiogenesis and Other Species of Fish

The muscle tissue of shark is active anti-angiogenically.

The anti-angiogenic activity of muscle tissue from other species of fish was compared with that of shark.

ANTI-ANGIOGENIC ACTIVITY OF EXTRACT FROM FISH

HOKI (NZ FISH)	24.7% inhibition at 200µg/ml
FISH OIL (Commercial)	88.2% inhibition at 100µg/ml
COD LIVER OIL	42.0% inhibition at 100µg/ml
SHELL FISH OIL (Abalone)	52.7% inhibition at 100µg/ml
SHARK FLESH OIL	79.8% inhibition at 5µg/ml
[OLIVE OIL]	No inhibition at 200µg/ml

Composition of Oil from Shark Muscle

In view of the strong anti-angiogenic activity associated with the oil extracted from shark muscle, analyses of its composition were performed.

Fatty Acid Composition (w/w %) of Blue Shark Flesh Extract

Fatty Acid	Retention Time (min)	Ethanol Extract
16:0	(9.40) 10.07	29.2
16:1	10.59	2.0
18:0	(13.74)14.85	12.6
18:1(n-9)	(14.24)15.53	16.8
18:1(n-7)	15.74	4.9
18:2(n-6)	17.16	0
20:1(n-11)	23.45	0
20:1(n-9)	23.60	0
20:4(n-6) AA	27.04	3.6
20:5(n-3) EPA	29.15	5.9
??	34.34	0
22:5(n-3)	37.53	5.0
22:6(n-3) DHA	39.10	20.0
Total Fatty Acid Content(%)		7.3

Phospholipid Composition (w/w %) of Blue Shark Flesh Extract

Phospholipid	Shift (ppm) (relative to PC)	Ethanol Extract
PG	1.24 - 1.27	2.2
PI	1.52 - 1.60	1.7
SM	0.79 - 0.83	6.9
PE	0.56 - 0.63	22.0[2]
MPE/LPC?	0.46 - 0.48	5.7
DPE?	0.19 - 0.28	5.0
AAPC	0.06	
PC	0	56.51[1]

[1] PC + AAPC
[2] PE + MPE/LPC

The lipid extract from the shark muscle has been called

MACOLIPIN

Inhibition of Angiogenesis

In vitro Aortic Ring Assay

50% Inhibition of angiogenesis is achieved at 5μg/ml

In vivo Mesenteric Window Assay

50% Inhibition of angiogenesis is achieved at 17mg/kg body weight

When macolipin is mixed with olive oil at a ratio of 1 of MACOLIPIN to 9 of olive oil and assayed for anti-angiogenic activity:

Inhibition of Angiogenesis

In vitro Aortic Ring Assay

50% Inhibition of angiogenesis is achieved at 20μg/ml
This is the equivalent of 2μg/ml of MACOLIPIN
The activity of the MACOLIPIN has doubled

In vivo Mesenteric Window Assay

50% Inhibition of angiogenesis is achieved at 25mg/kg body weight
This is the equivalent of 2.5mg/kg of MACOLIPIN
The activity of the MACOLIPIN is about 7 times higher

Mechanism of Action of Macolipin: Olive Oil

Preliminary experiments have shown that Macolipin: Olive Oil (9:1) has the following actions, which are relevant to its anti-angiogenic activity

* Macolipin/Olive oil (1:9) mixture has a moderate antagonistic activity on the stimulatory action of PDGF and IL-2.

* Macolipin/Olive oil (1:9) mixture strongly inhibits the pro-angiogenic effect of VEGF, FGF-2, and TGF-β.

* Macolipin/Olive oil (1:9) mixture can bind to both the R1 and R2 receptors for VEGF and thereby prevent VEGF binding to the cell surface.

* Macolipin/Olive oil (1:9) mixture may have a stronger affinity for the R2 receptor for VEGF.

* It is likely that Macolipin/Olive oil (1:9) mixture has multiple methods of inhibiting angiogenesis.

Methods

In vitro assay of angiogenesis (Aortic Ring Assay)

The aorta is removed from a rat and cleaned of adhering fatty and connective tissues before being cut into rings of approximately 3mm size. Fibrinogen is layered in the bottoms of wells of multi-well culture plates and allowed to gel by thrombin action. A ring is then layered on the top of each gel and a further layer of fibrin placed on this. The fibrinogen is prepared in MCDB131 medium supplemented with antibiotics. The double layer of fibrin is then overlaid with MCDB131 containing the test materials.

The gels are incubated at 37°C in an atmosphere of 3%CO_2/97% air. The rings are examined using an inverted microscope and the growth of micro vessels from their perimeters is observed. Digital pictures are taken of these every 2 days and the extent of micro vessel growth relative to the size of the ring is determined using NIH Image software. From this, the rate of growth of micro vessels can be determined for each well.

Each test substance is assayed in triplicate and the mean growth rate is calculated.

In vivo assay of angiogenesis (Mesenteric Window Assay)

Samples are evaluated for their ability to inhibit induced angiogenesis in the mesenteric windows of rats. For this experiment, the rats had their diet supplemented with the extracts for two weeks prior to the induction of angiogenesis. The rats had their water supply supplemented with the lipids at 0.167mg/ml. The water is available *ad lib* to the rats and the daily consumption is measured and therefore the dosage of lipid is recorded.

Compound 48/80 is injected intra-peritoneally twice daily into the rats for 4-5 days at increasing doses as described by Norrby et al. This involved 1μg Compound 48/80 per g body weight on day 1, 2μg/g body weight on day 2, 3μg/g body weight on day 3, 4μg/g body weight on day 4 and 5μg/g body weight on day 5. Sixteen days after the commencement of this, the vasculature of the rats is highlighted with India ink, the rats are sacrificed and the mesenteric windows excised, laid on glass slides and dried. These are scanned digitally and the images used for determining the extent of vascularisation of each window. For each animal approximately 20 consecutive windows are used for the calculation.

Each rat is weighed at the start of the experiment, at the time of commencement of Compound 48/80, at the conclusion of this and at the end of the experiment.

Preparation of Immune Complexes

The reaction samples are prepared as indicated in Table 3, react in room temperature with shaking for 2 hours (for No. 5 and No. 9, VEGF are added after 1 hour reaction).

TABLE 3.

PREPARATION OF IMMUNO-COMPLEX OF VEGF AND THEIR RECEPTORS IN THE PRESENCE/ABSENCE OF MACOLIPIN: OLIVE OIL

Sample Number	VEGF (5μg/ml) (μl)	VEGF-R1 (10μg/ml) (μl)	VEGF-R2 (10μg/ml) (μl)	Macolipin: Olive oil (100μg/ml) (μl)	Olive Oil (100μg/ml) (μl)	4 X Binding Buffer* (μl)	DDW (μl)	Total Volume (μl)
1	20				20	50	110	200
2		100			20	50	30	200
3	20	100			20	50	10	200
4	20	100		20		50	10	200
5	20 (Add at 1 hr later)	100		20		50	10	200
6			100		20	50	30	200
7	20		100		20	50	10	200
8	20		100	20		50	10	200
9	20 (Add at 1 hr later)		100	20		50	10	200

* 4 X Binding buffer: 4 mM CaCl2, 0.4 mM CuSO4, 0.5% Tryptone in DMEM

SDS-Polyacrylamide Gel Electrophoresis and Immunoblotting

For the immunoblotting detection of VEGF-R1 and VEGF-R2, 5% polyacrylamide gels are used and for the immunoblotting of VEGF, 10% gels are used.

Aliquots (20μl) of the incubation mixtures are loaded into the lanes of the gel and electrophoresed at 200V for 1 hour. The separated proteins are transferred electrophoretically

from the gel to a PVDF membrane using a BioRad semi-dry transfer apparatus. Following the transfer, the membrane is blocked by incubating the membrane in blocking buffer (0.05% Tween20 in PBS containing 3% non-fat dry milk).

After washing, the membrane is incubated while shaking with the primary antibody for 2 hours at room temperature. It is then washed 3 times (5min for each wash) with the washing buffer. The membrane is then incubated by shaking with Horseradish peroxidase-conjugated secondary antibody for 1 hour at room temperature. The membrane is again washed 3 times with washing buffer.

The membrane is then developed using the ready-to-use solution TMB by incubating it in this solution and stopping the colour development by rinsing with deionized water.