Abstract

An ethanol extract from shark muscle has been shown to have potent angiogenic activity when mixed together with olive oil in a ratio of 1part extract to 9 parts olive oil. This mixture has been given the trade name SuperMaco (SMO).

The anti-angiogenic activity was evident in both in vitro and in vivo studies in rats. When combined with the known angiogenic stimulatory growth factors Vascular Endothelial Growth Factor (VEGF), Platelet Derived Growth Factor (PDGF), Transforming Growth Factor-Beta (TGF-β) and Fibroblast Growth Factor -2 (FGF-2), SMO completely eliminated the stimulation induced by these growth factors. Possible mechanisms for this activity are that SMO is competing for receptor sites on the endothelial cell surface by preferential binding to those sites or by binding to the growth factors themselves.

Studies were conducted using soluble forms of VEGF-R1 and VEGF-R2 and it was found that SMO inhibited complex formation of the VEGF with both receptors. This inhibitory effect by SMO was enhanced when SMO was pre-incubated with VEGF receptors.

In another study, the effect of SMO on VEGF receptor phosphorylation using human umbilical vein endothelial cells (HUVEC) and a human breast carcinoma cell line (MDA-MB-231) was investigated. The in vitro cell proliferation assay indicated that SMO reversed the increase in VEGF promoted cell proliferation in both HUVEC and MDA-MB-231 cells. Using Western Blot analysis it was observed that SMO significantly inhibited VEGF promoted VEGF receptor-1 , VEGF receptor-2 and tyrosine phosphorylation in HUVEC and MDA-MB-231 cells in a dose related manner. These results suggest that SMO appears to block the binding of VEGF with the receptors and inhibit the signal transduction pathways.

Abstract (cont'd)

A Clinical Study

To investigate the possibility that these anti-angiogenic functions of SMO might have clinical activity against immunological disorders such as psoriasis an initial study was conducted on 10 patients with clinically assessed psoriasis. The patients were measured against a Physician Global Assessment (PhGA), Patient Global Assessment (PaGA) and EuroQol-5D. Each patient took 10 capsules of SMO 3 times each day (a total of 30 capsules per day) with the treatment continuing for 12 weeks.

The results indicated that the treatment with SMO improved all three parameters (PhGA, PaGA and EuroQol-5D scales) for these patients. This suggests that suppression of the above mentioned receptors by SMO is also effective against immunological disorders such as psoriasis.

Introduction

In 1971, Dr. Judah Folkman first proposed the hypothesis that tumor growth is angiogenesis dependent. Angiogenesis is essential for the growth of most primary tumors and their subsequent metastasis. Tumors larger than 1-2mm, thought to recruit the neighboring host mature vasculature to begin sprouting new blood vessel capillaries which grow toward and infiltrate tumor mass to supply sufficient nutrients and oxygen for tumor cells. Because angiogenesis inhibitors are relatively less toxic than conventional chemotherapy and have a lower risk of drug resistance, inhibiting angiogenesis would therefore seem to be a reasonable approach to prevent or treat cancer.

From around 1990, shark cartilage has been widely promoted as a possible therapeutic preparation for the treatment of solid tumors through its proposed anti-angiogenic action. However, it was not until 1997 that there was evidence that the oral administration of shark cartilage was anti-angiogenic. Subsequent intensive studies showed that the lipid fraction of the shark cartilage was at least partly responsible for this in vivo antagonistic action (Davis PF et al, unpublished data). It subsequently became apparent that lipid-rich preparations from several shark organs were active with that from cartilage, muscle and spinal cord being particularly active (Davis PF et al, unpublished data). Lipid rich extracts from other species of fish were also anti-angiogenic, however, the potencies were less than shark lipid.

The ethanol extract from shark muscle has been shown to have potent angiogenic activity when mixed together with olive oil in a ratio of 1part extract to 9 parts olive oil. This mixture has been given the trade name SuperMaco (SMO).

The purpose of this study is to determine the mechanism of SMO for anti-angiogenesis. Preliminary results of clinical evaluation of SMO for psoriasis patients are also shown in this paper.

Shark lipid extract with olive oil (SuperMaco)

The ethanol extract of shark muscle (macolipin) contains high ratio of phospholipids (such as phosphatidylcholine) and omega-3 fatty acids (such as DHA). Olive oil showed synergistic effects on anti-angiogenic activity of the shark lipid extract. The macolipin mixed with olive oil in the ratio of 1:9 (SMO) showed 100 times more potent inhibitory activity comparing with shark cartilage determined by the aortic ring assay in vitro and 30 times more potent by the mesenteric window assay in rats.

Major Fatty Acids in macolipin
(% of total fatty acids)

Palmitic Acid	(16:0)	29.2
Stearic Acid	(18:0)	12.6
Oleic Acid	(18:1)(n-9)	16.8
EPA	(20:5)	5.9
DHA	(22:6)	20.0

Major Phopholipids in macolipin
(% of total phospholipids)

Phosphatidylathanolamine	22.0
Phosphatidylcholin	56.5
Sphingomyelin	6.9
Phosphatidylinositol	1.7

Methods

In vitro assay of angiogenesis (Aortic Ring Assay) (Fig. 1)
A length of aorta was removed from a rat and be cut into rings of approximately 3mm size. Fibrinogen was layered in the bottoms of wells of 24-well culture plates and allowed to gel by thrombin action. A ring was then layered on the top of each gel and a further layer of fibrin placed on this. The fibrinogen was prepared in MCDB131 medium. The double layer of fibrin was then overlaid with MCDB131 containing the test materials.The gels were incubated at 37°C. Digital pictures were taken of these after seven days and the extent of microvessel growth relative to the size of the ring was determined using NIH Image software.

SDS-Polyacrylamide Gel Electrophoresis and Immunoblotting (Figs 2)
For the detection of VEGF-R1 and VEGF-R2 by immunoblotting, 5% polyacrylamide gels were used and for the immunoblotting of VEGF, 10% gels were used. Aliquots (20µl) of the incubation mixtures were loaded onto the lanes of the gel and electrophoresed at 200V for 1 hour. The separated proteins were transferred electrophoretically from the gel to a PVDF membrane, the membrane was blocked and incubated with the primary antibody. The membrane was then developed using the ready-to-use solution TMB by incubating it in this solution and stopping the color development by rinsing with deionized water.

Detection of tyrosine phosphorylation in HUVEC and MDA-MB-231 cells by Western Blot Analysis (Figs 3 and 4)

Cell lysate preparation:
HUVEC and MDA-MB-231 cells were serum starved for 24 hours in their culture medium containing 0.1% serum. After pre-treatment with 100 nM sodium orthocanadate in PBS for 30 minutes, the cells were incubated with 50 ng/ml of VEGF in the presence of series concentration of SMO for an additional 30 minutes. The cells were then washed ice-cold PBS. Cell lysates were extracted.

Methods (cont'd)

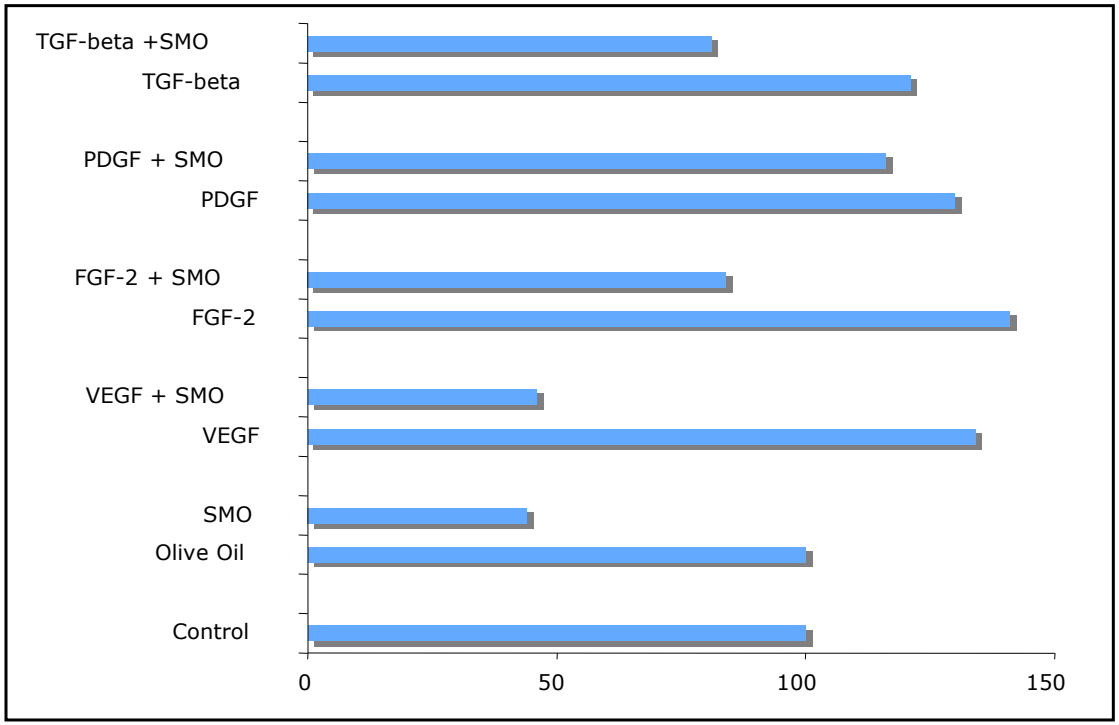
Detection of tyrosine phosphorylation in HUVEC and MDA-MB-231 cells by Western Blot Analysis (cont'd)

Preparation of immune complex: One ml of cell lysate was pre-cleared by incubation with 10 ml of a 50% suspension of anti-mouse IgG agarose beads for 5 minutes on ice. The beads were pelleted by centrifugation at 3,000 x g, 4°C for 0.5 minutes and the supernatant was transferred to a new tube. The pre-clearing was repeated one time. The anti-mouse IgG agarose beads were washed three times with Wash Buffer before being added to the lysates.

Immunoprecipitation: Five microliter of mouse anti-phospho-tyrosine antibody was added to 500 ml of cell lysate and the mixture was rocked for 16 hours at 4 °C. Twenty ml of anti-mouse IgG agarose beads (50% suspension) was then added and the mixture was rocked at 4 °C for an additional 2 hours. The anti-mouse IgG-absorbed complexes were centrifuged at 3,000 x g, 4 °C for 0.5 minutes, resuspended in Wash Buffer by trituration with a glass Pasture pipette, and the re-pelleted. The complexes were washed a total of three times with Wash Buffer, and then suspended in 50 ml of phosphate-buffered saline and transferred to a new tube before final centrifugation. The washed pellet was suspended in 50 ml of 2xSDS Gel Sample Buffer. Anti-mouse IgG agarose was pelleted and the prepared supernatants were collected and stored at -20 °C for Western blot analysis.

Western blot analysis: Primary antibodies: Monoclonal Anti-phasphotyrosine antibody was used for detection of phosphorylated tyrosine. Monoclonal Anti-VEGF Receptor-1 and monoclonal anti-VEGF receptor-2 antibody were used for detection of VEGF-receptor-1, and VEGF-receptor-2. Second antibody: anti-mouse IgG-HRP antibody was used as the second antibody. According to the different molecular sizes, different concentrations of acrylamide of SDS-polyacrylamide gels were prepared: 10% for phosphorylated tyrosine, 8 % for VEGF Receptor-1 and 6% for VEGF Receptor-2. Ten µl of prepared sample was applied. The membranes were incubated in antibody solution with the monoclonal anti-phosphotyrosine antibody, anti-VEGF Receptor-1 and monoclonal anti-VEGF receptor-2 antibody, respectively for over night at 4°C with shaking. The membranes were detected by using ECL Reagent and exposed to an X-ray film.

Inhibitory Effects of SMO on Cytokines-stimulated Angiogenesis



SMO, SuperMaco; VEGF, Vascular Endothelial Growth Factor; FGF-2, Fibroblast Growth Factor – 2; TGF-beta, Transforming Growth Factor-beta; PDGF, Platelet-Derived Growth Factor.

Fig. 1 Inhibitory Effects of SMO on Cytokines-stimulated Angiogenesis

Samples added into wells as following: Control, 0.2% EtOH; Olive Oil, 30 μg/ml; SMO, 30 μg/ml; VEGF, 32.6 ng/ml; FGF-2, 10 ng/ml; PDGF, 6 ng/ml and TGF-beta, 0.08 ng/ml. The calculation of angiogenesis in the control was 100% growth, the growth of angiogenesis in all samples were compared with the 100% growth of olive-oil control, the growth of angiogenesis in samples which were more than 100% were defined as stimulation.

Reduction of VEGF – VEGF Receptors complex formation by SMO

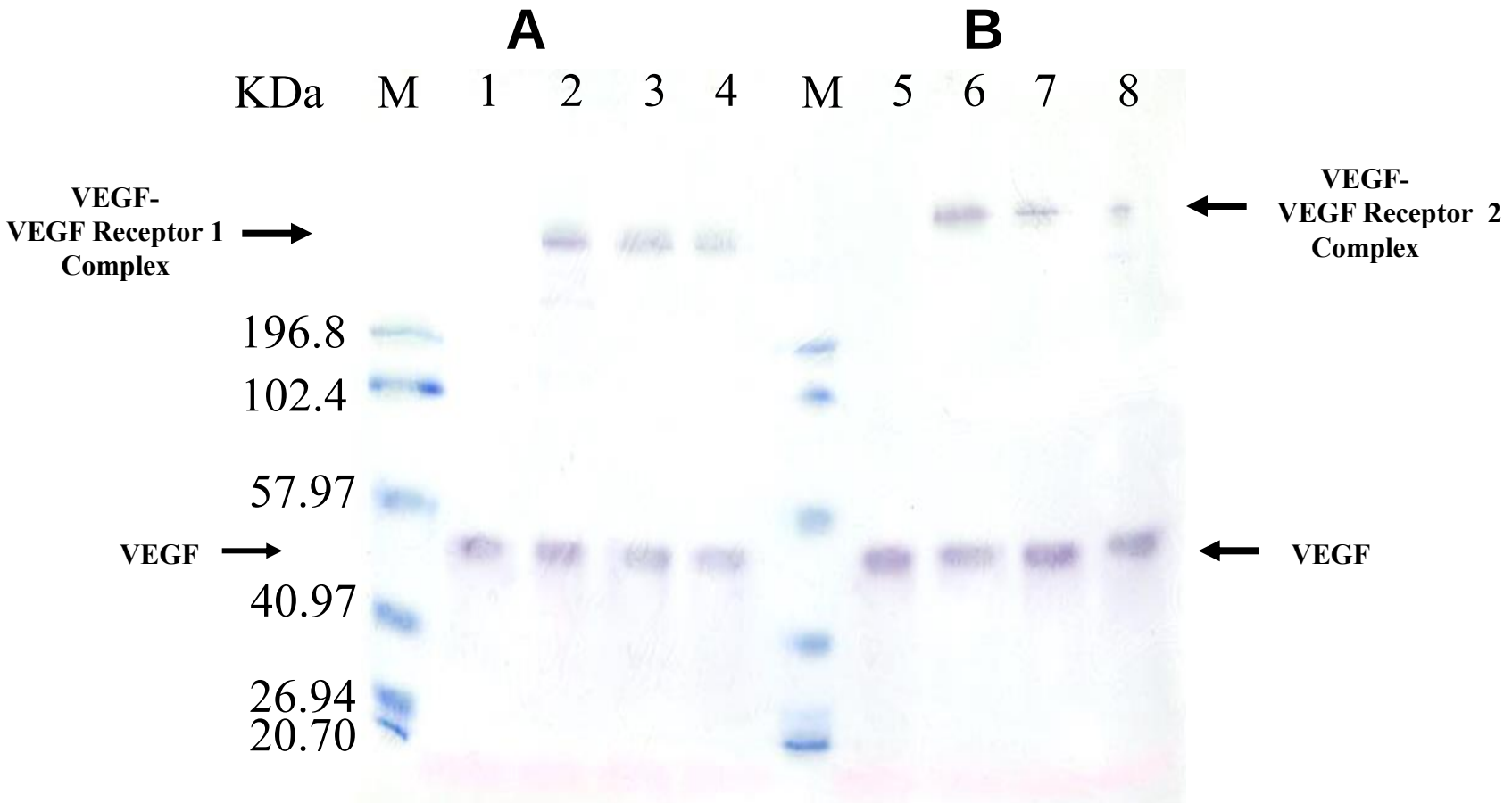


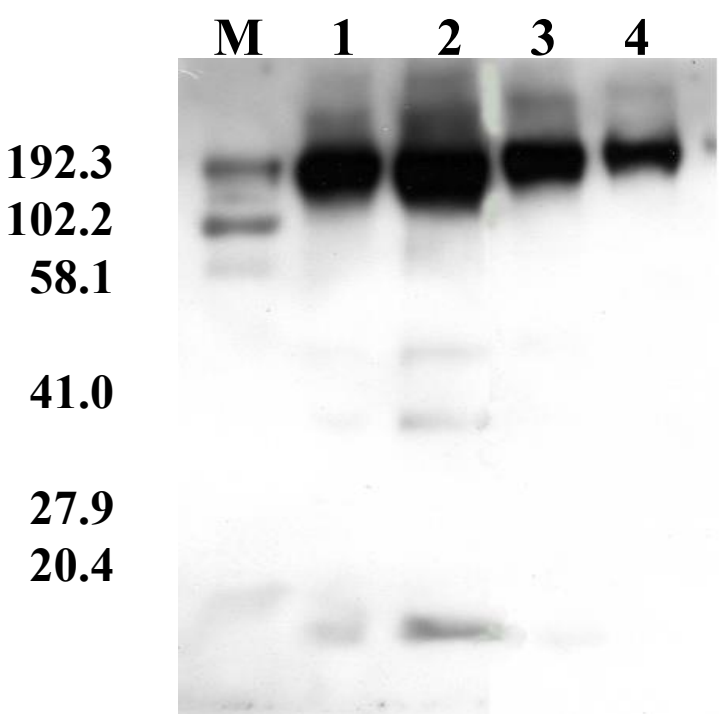
Fig. 2 Reduction of VEGF – VEGF Receptors complex formation by SMO

Incubation with VEGF-R1 (A. Lanes 1 - 4) and with VEGF – R2 (B. Lanes 5 - 8).
Immunoblotting with anti-VEGF (A and B. Lanes 1 - 8). Lane M, Molecular weight markers.
The following incubation mixtures were loaded onto the gel:

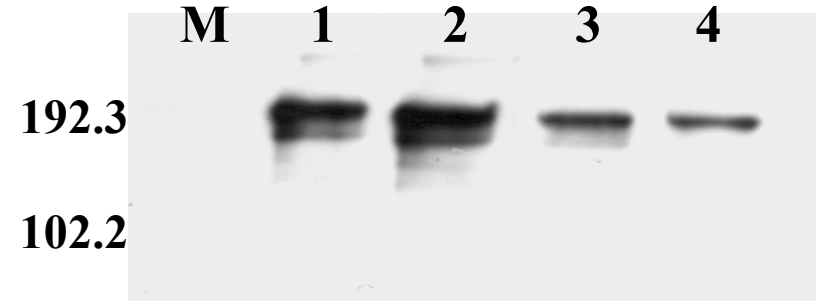
A. Lane 1, VEGF-R1; Lane 2, VEGF-R1 + Olive Oil + VEGF; Lane 3, VEGF-R1 + SuperMaco (SMO) + VEGF; Lane 4, VEGF-R1 pretreated with SMO before VEGF is added;

B. Lane 5, VEGF-R2; Lane 6, VEGF-R2 + Olive Oil + VEGF; Lane 7, VEGF-R2 + SMO + VEGF; Lane 8, VEGF-R2 pretreated with SMO before VEGF is added.

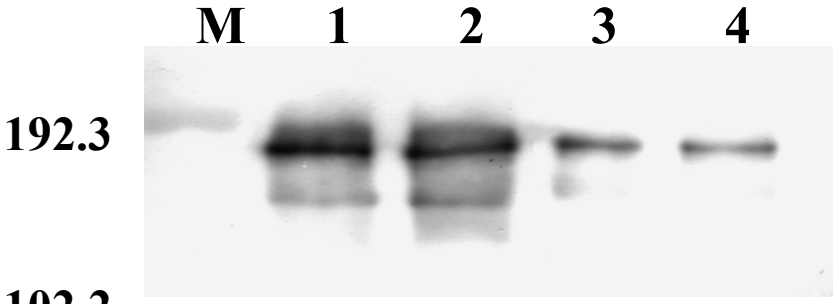
Effects of SMO on VEGF induced Tyrosine Phosphorylation in HUVEC Cells



Total Tyrosine Phosphorylation



VEGF Receptor 1 Tyrosine Phosphorylation



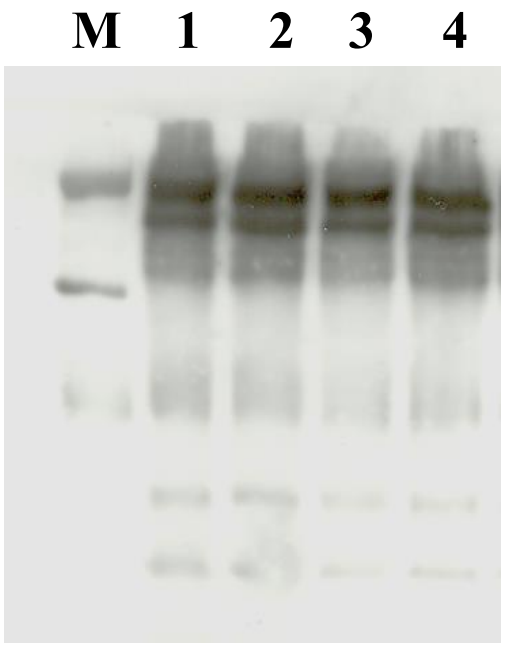
VEGF Receptor 2 Tyrosine Phosphorylation

1, Olive Oil Control	2, VEGF+Olive Oil Control
3, VEGF+SMO 50ug/ml	4, VEGF+SMO 10ug/ml

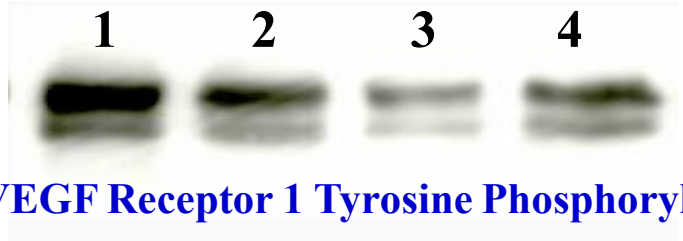
Fig.3 Effects of SMO on VEGF-induced tyrosine phosphorylation.

The HUVEC were incubated with/without 50 ng/ml of VEGF in the presence/absence of different concentration of SMO for an additional 30 minutes. The cell lysates were prepared. The phosphorylated tyrosine kinase proteins were immunoprecipitated with anti-phospho-tyrosine antibody and detected with anti-phospho-tyrosine antibody (total phosphorylated tyrosine), anti-Flt1 antibody (VEGF receptor-1, Flt1) and anti-KDR antibody (VEGF receptor-2) by Western blot analysis.

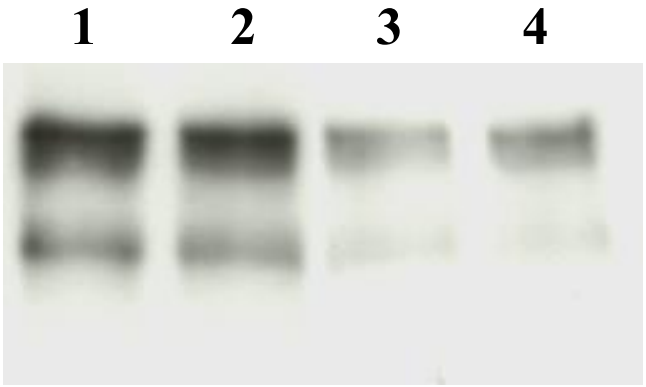
Effects of SMO on VEGF induced Tyrosine Phosphorylation in MDA-MB-231 Cells



Total Tyrosine Phosphorylation



VEGF Receptor 1 Tyrosine Phosphorylation



VEGF Receptor 2 Tyrosine Phosphorylation

1, Olive Oil Control	2, VEGF+Olive Oil Control
3, VEGF+SMO 50ug/ml;	4, VEGF+SMO 10ug/ml;

Fig. 4 Effects of SMO on VEGF-induced tyrosine phosphorylation.

The MDA-MB-231 cells were incubated with/without 50 ng/ml of VEGF in the presence/absence of different concentration of SMO for an additional 30 minutes. The cell lysates were prepared. The phosphorylated tyrosine kinase proteins were immunoprecipitated with anti-phospho-tyrosine antibody and detected with anti-phospho-tyrosine antibody (total phosphorylated tyrosine), anti-Flt1 antibody (VEGF receptor-1, Flt1) and anti-KDR antibody (VEGF receptor-2) by Western blot analysis.

Study of Psoriasis with SuperMaco Adjuvant Therapy

SuperMaco (SMO) and Psoriasis study rational

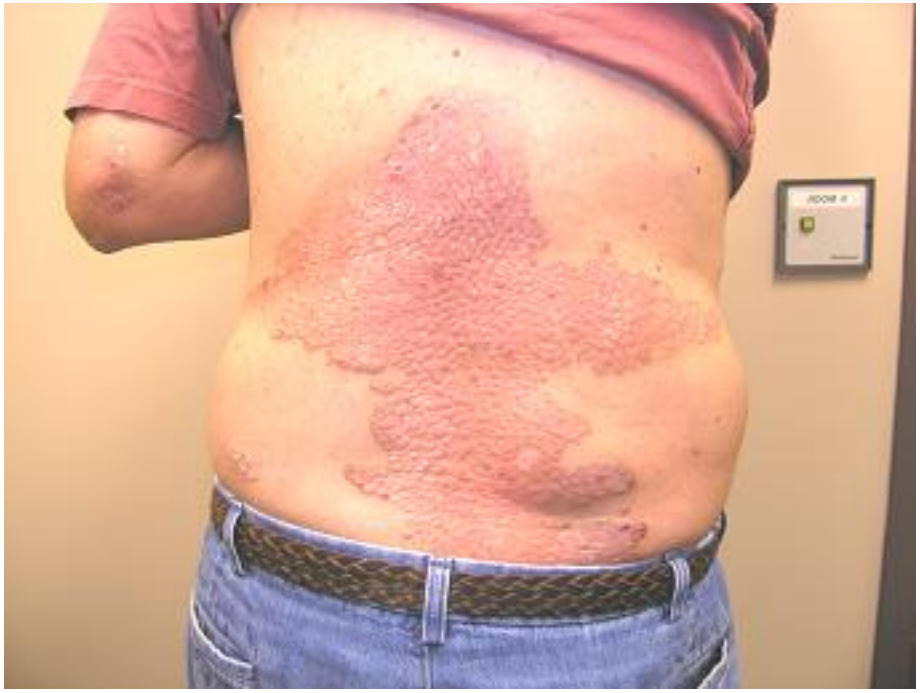
- ✧ SuperMaco inhibits VEGF, Fibroblast Growth Factor and Transforming Growth Factor Beta
- ✧ Psoriatic plaques demonstrate elevated levels of VEGF, FGF and TGF-beta

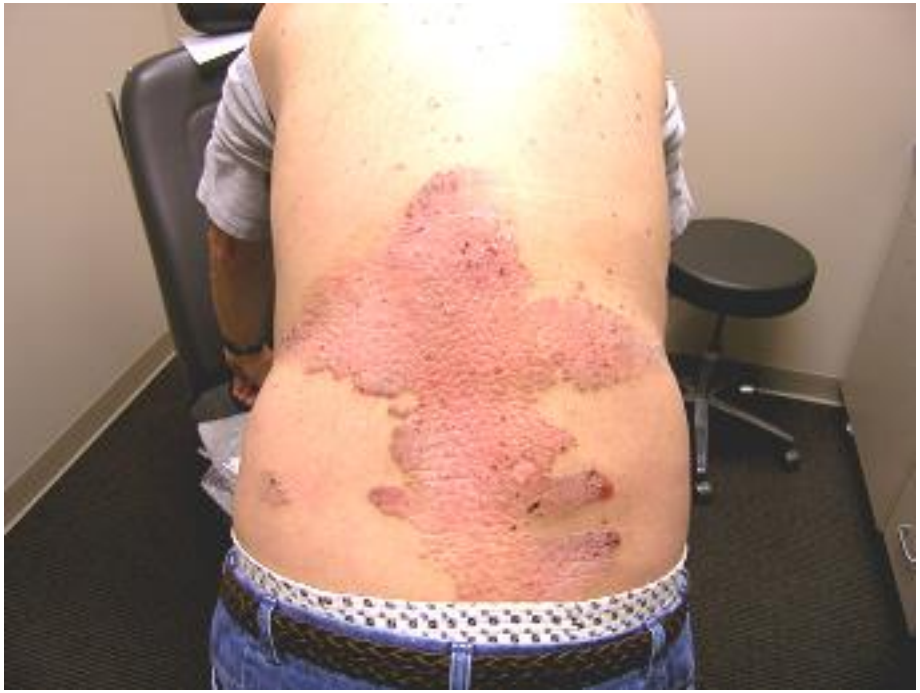
Study design

- ✧ Case Control Study of psoriatic patients at the Grekin Skin Institute in Wyandotte, Michigan USA.
- ✧ Patients who have failed or responded poorly to current therapies are eligible for enrollment
- ✧ Base line photographs, Patient Global Assessment, Physician Global Assessment, and EuroQol-5D (feeling thermometer) were obtained at baseline and every four weeks for twelve weeks.
- ✧ Ten study participants take 10 SuperMaco (SMO) pills three times a day for a total of 30 pills per day

Case report 1

42 y/o Male with severe plaque psoriasis present for greater than 20 years. Patient has severe itch and joint pain. Patient had failed previous topical therapies, UV therapy and was denied biologics by his insurance company





Before

Baseline PGA: 5

Baseline Patient Assessment: 5

EuroQoL-5D: 60

After (4 weeks)

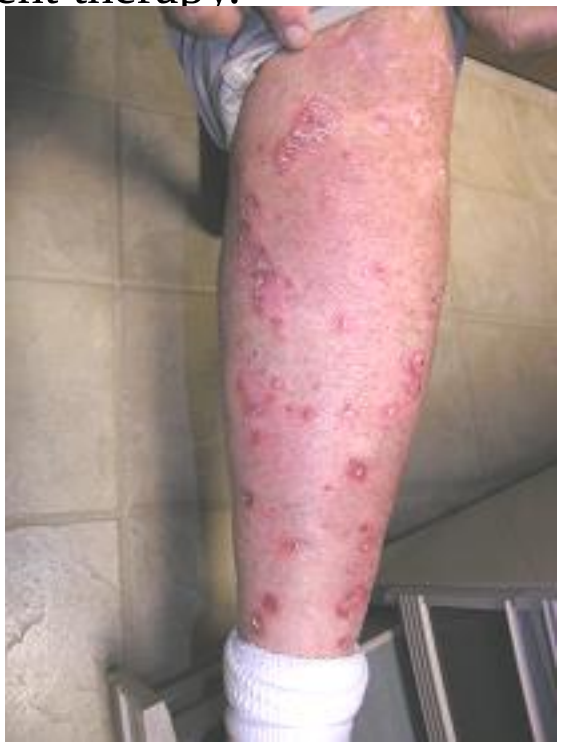
PGA: 4

Patient Assessment: 4

EuroQol-5D: 75

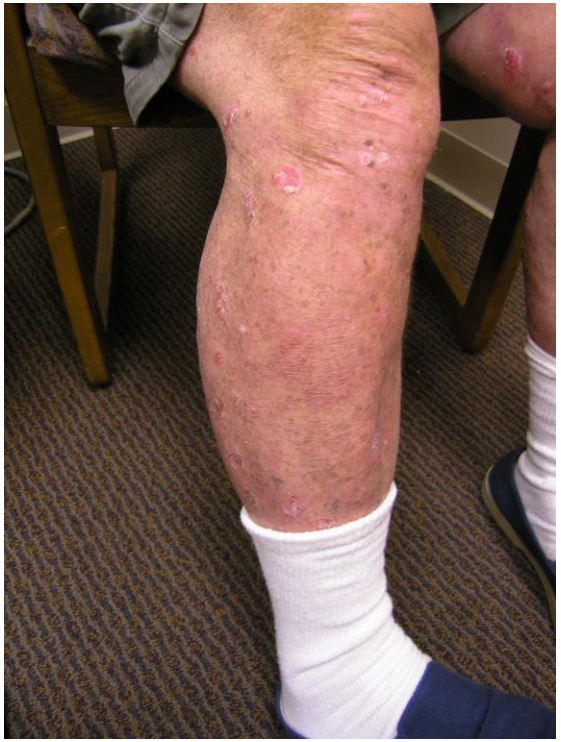
Case Report 2

66 y/o white male with plaque psoriasis on lower extremities for approximately 30 years. Patient had tried multiple topical steriods and narrow band UVB with mild to moderate improvement but on day of enrollment presented with flare of psoriasis despite current therapy.



Before

Baseline PGA: 3.5
Baseline Patient Assessment: 4
EuroQoL-5D: 68

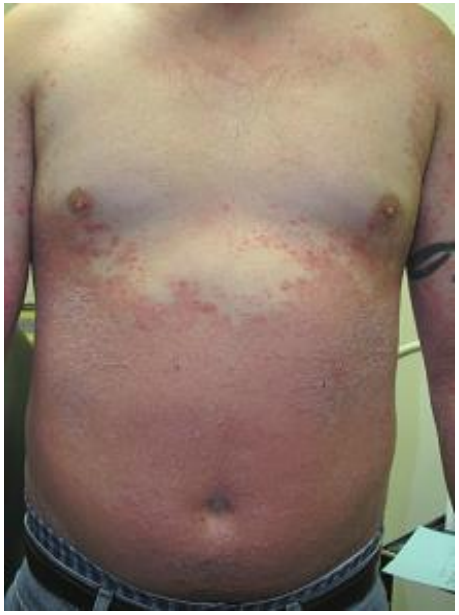


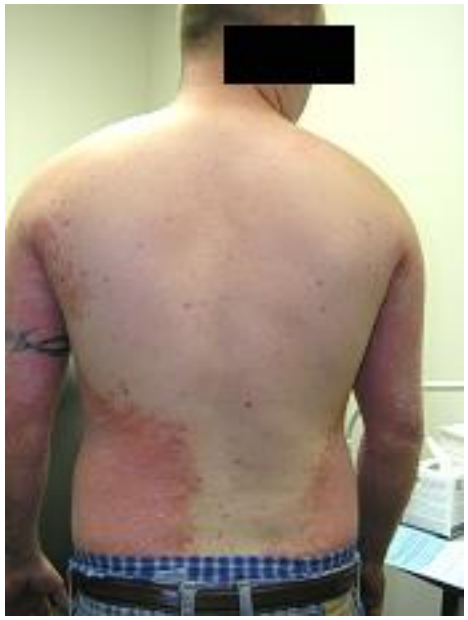
After (8 weeks)

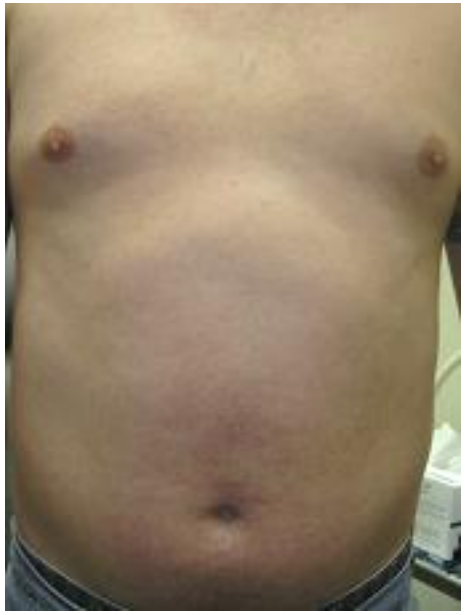
PGA: 2
Patient Assessment: 2
EuroQoL-5D: 80

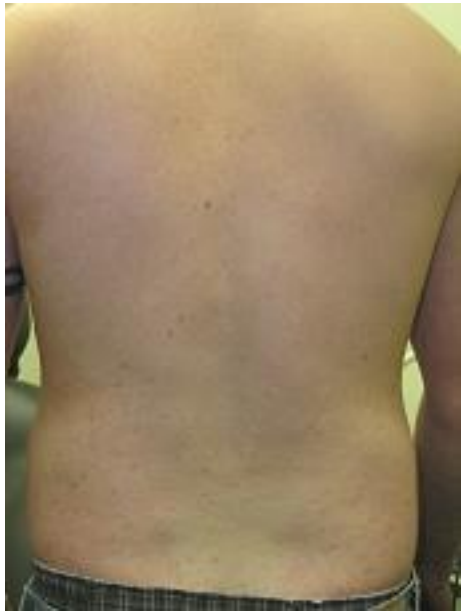
Case Report 3

26 y/o male who had severe psoriasis on approximately 65% on his BSA for last 8 years. This patient had failed two biologic therapies, as well as, light therapy and topical steroids. SuperMaco is administrated alone in this therapy.









Before

Baseline PGA: 5

Patient Assessment: 5

EuroQoL-5D: 30

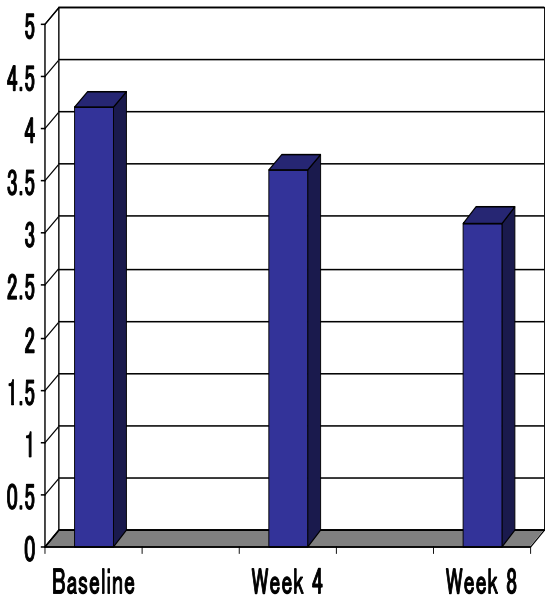
After (12 weeks)

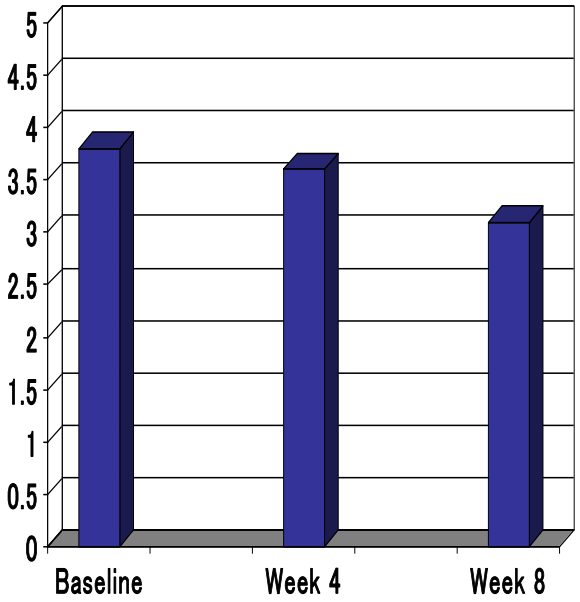
PGA: 0

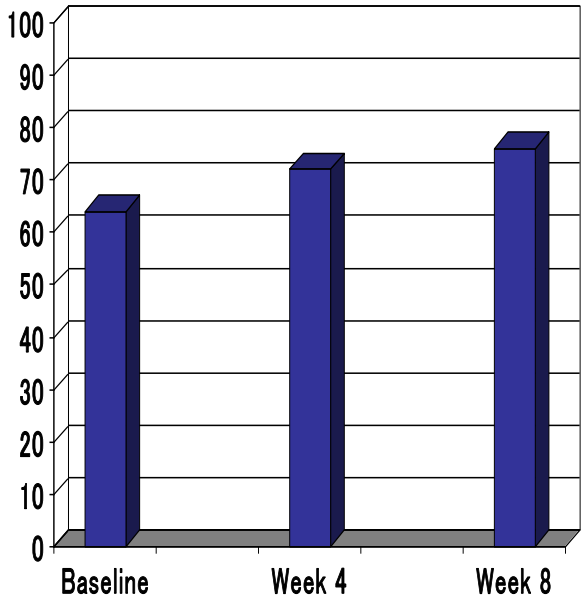
Patient Assessment: 0

EuroQoL-5D: 95

Preliminary Results of SMO Study for Psoriasis
(Mean scores for 10 patients)







Physician Global Assessment

A validated measure of psoriasis activity. Rates psoriasis based on induration, erythema and scaling based on a scale of zero (no evidence of disease) to 5 (induration is 2.5mm or more, erythema is dusky to deep red in color, scale is severe and thick)

Patient Assessment

Scale from zero to five, patients rates self on overall assessment of psoriasis, joint pain and itch. Similar to Physician Global Assessement with zero being no evidence of disease and five being severe disease

EuroQoL-5D

(Feeling Thermometer)

EuroQoL is a measure of overall quality of life. A quality of life is measured by patient on a scale from zero to 100. Zero being worst imaginable health and 100 being best imaginable health

Conclusions

1. SMO inhibited various cytokines such as VEGF, FGF-2, PDGF and TGF-beta stimulated rat aortic ring angiogenesis in vitro angiogenesis assay.
2. SMO reduced VEGF-VEGF-receptor-1 and receptor-2 complex formation in vitro (cell free system) detected by immunoblotting assay
3. SMO inhibited total tyrosine phosphorylation in HUVEC and in MDA-MB-231 cells detected by western blot analysis.
4. SMO inhibited VEGF-receptor-1 and VEGF receptor-2 tyrosine phosphorylation in HUVEC and in MDA-MB-231 cells by western blot analysis.
5. Study of psoriasis with SMO adjuvant therapy has been conducted at Grekin Skin Institute, MI
6. SMO clinically reduced the size of psoriatic plaques and improved the subjects quality of life.