POSEIDON

ANTI - INFLAMMATORY

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A derivative of menthol has been discovered by scientists at Poseidon Sciences to possess anti-inflammatory and anti-angiogenic activity. This carbonic acid derivative and analog of menthol, known as menthol propyleneglycol carbonate (MPC), showed excellent properties in reducing inflammation and inhibiting the formation of blood vessels (the anti-angiogenic response).

The new application for this FEMA GRAS ("generally regarded as safe" by the US FDA) compound offers new treatment modalities for inflammation and other diseases, such as eczema, psoriasis, obesity and cancer.

Overview

Research into the use of naturally-occurring chemical compounds for application as topical medication is motivated in part, by growing public concern over the possible health risks associated with products of this type that contain synthetic active agents. Consequently, efforts continue toward the development of safe and effective therapeutic agents based on natural compounds.

Menthol is a natural product which is obtainable from peppermint oil and other mint oils. Menthol and its various analogs, such as isopulegol, N-ethyl-p-menthane-3-carboxyamide and p-menthane-3, 8-diol, are used in commerce as cooling agents. These compounds impart a cooling sensation to a variety of products such as cosmetics, perfumes, personal care products, oral hygiene products, confectionery, cigarettes, cough drops, nasal inhalants and the like.

The discovery by Poseidon scientists that the menthol analogue, menthol propyleneglycol carbonate (MPC) possessed inhibitory effects in TPA-induced inflammatory response and significant anti-angiogenic effect is noteworthy. By comparison menthol showed no appreciable anti-inflammatory effect under the same test conditions.

MPC is a GRAS (generally regarded as safe) material known by number 3806 in the U.S. FEMA (Flavor and Manufacturers' Association) list and as number 444 on the JECFA (Joint FAO/WHO Expert Committee on Food Additives) list. The latest JECFA assessment conducted in 1999 of menthol and its derivatives including MPC reaffirmed this compound's safety as an ingredient in food and cosmetics

This report describes the anti-angiogenic and anti-inflammatory activities of MPC *in vitro* and *in vivo*.

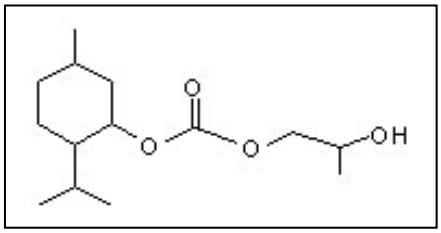

Fig. 1. Structure of MPC

MPC as an Anti-Inflammatory Agent

The mouse ear edema model is a standard animal test procedure to document the anti-inflammatory effect of an agent. In this test, edema was induced in mice through the topical application of 10µl of TPA (Tetradecnoylphorbol acetate) in acetone (2.5µg/ear) to both the inner and outer surface of one ear of each mouse. Each test compound, diluted with acetone to a concentration of 10% was applied topically to the inflamed mouse ear immediately after TPA application, so as to deliver 2.5 mg/ear. The reference drug, indomethacin (0.5mg/ear), was administered as a positive control. The thickness of each ear was measured before treatment and 4 hours after induction of inflammation, using a micrometer (Mitutoyo Co.). Anti-inflammatory effect was expressed as the reduction in ear thickness with respect to the control group.

When menthol and its isomers were tested for anti-inflammatory effects using the mouse edema assay, we observed no significant effects. However, when MPC was tested in the same assay, a dramatic inhibition of TPA-induced mouse ear edema was found. This effect was further tested by the topical application of MPC (referred in this study as HR-008) at 2% and 10%. The data are presented in Figure 2.

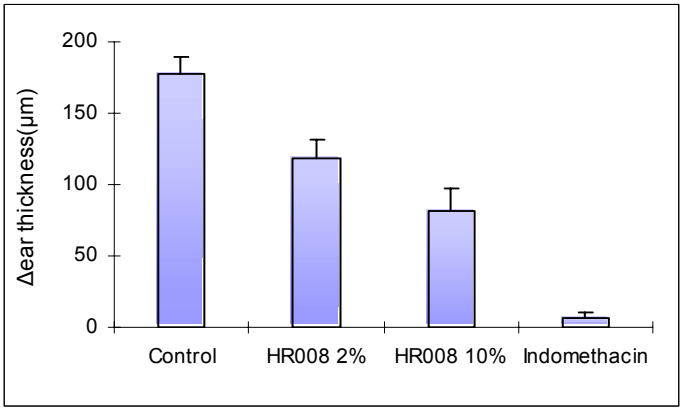

Fig. 2. Inhibition of TPA induced edema (ear thickening) by topical application of MPC on the mouse ear.

The study showed approximately a 30% reduction of the ear thickness by the application of a 2% solution of MPC in ethanol, and a 50% reduction using a 10% solution of MPC.

MPC as an Angiogenic Inhibitor

The effect of MPC was studied by culturing aortic explants in three-dimensional matrix gels according to the procedure of Kruger and Figg (Kruger E.A. and Figg, W.D. Protein binding alters the activity of suramin, carboxyamidotriazole, and UCN-01 in an *ex Vivo* Rat Aortic Ring Angiogenesis Assay. Clinical Cancer Research, 7:1867-1872, 2001).

Thoracic aortas were excised from 8-week-old male Sprague Dawley rats and the fibroadipose tissue removed. The aortas were sectioned into 1-mm-long cross-sections, rinsed with Human Endothelial-SFM (GIBCO), placed on the Matrigel-coated wells, covered with an additional 50 µl Matrigel, and allowed to gel for more than 30 min at 37ºC, 5% CO_2. All the rings were cultured in Human Endothelial-SFM (GIBCO) supplemented with 200µl/ml of ECGS (Endothelial Cell Growth Supplement, Sigma) as an angiogenesis inducer. Racemic menthol propylene-carbonate diluted with ethanol was added to the culture medium at final concentrations of 1µM, 10µM, and 100µM. Ethanol alone (1%) was added to the culture medium as a vehicle control. The area of angiogenic sprouting was calculated using Image-Pro Plus software (Media Cybernetics).

The quantitation by image analysis of the dose-response activity of MPC is shown in Table I. Microvessel densities are reported in square pixels. All assays were performed by using 5 aortic rings per sample. Aortic rings were photographed on day 10.

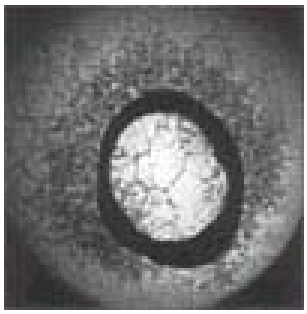

Fig. 3. The outgrowth of blood vessels from the aortic ring in the control.

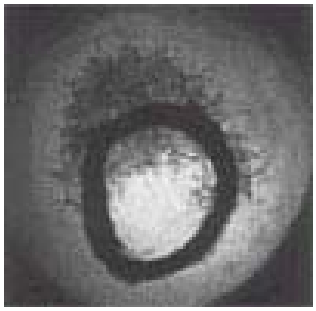

Fig. 4. The outgrowth of blood vessels is prevented by the addition 10µM of MPC.

Table I. The inhibitory effect of MPC on microvessel outgrowth in the rat aortic ring.

Concentration (µM)	Microvessel Density (pixel 2)	% inhibition
0	15.8 ± 4.0	0
1	13.4 ± 4.1	15
10	12.2 ± 2.5	22
100	10.6 ± 3.8	33

The data in Table I and in Figures 3 and 4 show that MPC exerted significant anti-angiogenic activity in a dose dependent manner.

MPC acts via Nontoxic Mechanisms

A cell cytotoxicity assay was performed to evaluate the cytotoxic effect, if any, MPC on normal cells.

Ha-CaT cells (5 X $10^{3)}$ were plated in each well of 96 well plates and MPC was added at various concentrations. The plates were incubated for another 48 hours. The viable cells were measured using an XTT Cell Proliferation Kit (Roche). More than 70% of Ha-CaT cells were viable even at a high concentration of 100µM. The results of this experiment are shown in Table II.

Table II.

Concentration (µM)	Cell Viability (%)
0.1	100
1.0	100
5.0	96.5
10.0	96.3
50.0	81.8
100	70.4

These data show that MPC is nontoxic against normal cells.

MPC Does Not Inhibit Proliferation of Endothelial Cells

An experiment was also performed to determine the effect of MPC on HUVE cell proliferation. HUVE (Human Umbilical Vein Endothelial) cells were isolated from human umbilical cord veins through a method of Jaffe et al. (Jaffe, E. A., Nachman, R. L., Becker, C. G., and Minick, C. R. Culture of Human endothelial cells derived from umbilical veins. J. Clin. Invest. 52: 2745-2756, 1973).

HUVE cells were confirmed by immunostaining with antibody against factor VIII. The cells were grown in M199 medium (Gibco BRL, Grand Island, NY) supplemented with 10% fetal bovine serum, 100units/mL penicillin, 100µg/mL streptomycin, 50 µg/ml endothelial cell growth supplement, and 5 units /ml heparin at 37 °C in an atmosphere of 5% CO_2-95% air.

$1x10^4$ HUVE cells were plated in each well of 96 well plates and five different concentrations ranging from 1 to 100 of MPC were tested in the presence of bFGF used as maximum proliferation control. Cells were cultured for an additional 48 hours, and relative cell numbers in each well were determined by XTT Cell Proliferation Kit (Roche).

Table III. Effect of MPC on human umbilical vein endothelial

	No bFGF	bFGF only	MPC-r* 100µM	MPC-r 50µM	MPC-r 10µM	MPC-r 5µM	MPC-r 1µM
Average	0.2283	1.0830	0.9121	1.0621	1.0425	1.0305	1.0623
Proliferation (%)	0.0	100.0	80.0	97.6	95.3	93.9	97.6

· MPC-r = racemic menthol propyleneglycol-carbonate

The data in Table III and in Fig. 5 show that even up to 100µM of MPC had weak inhibition of HUVE cell proliferation. The results demonstrate that this compound does not have detrimental effects on the normal proliferation of endothelial cells.

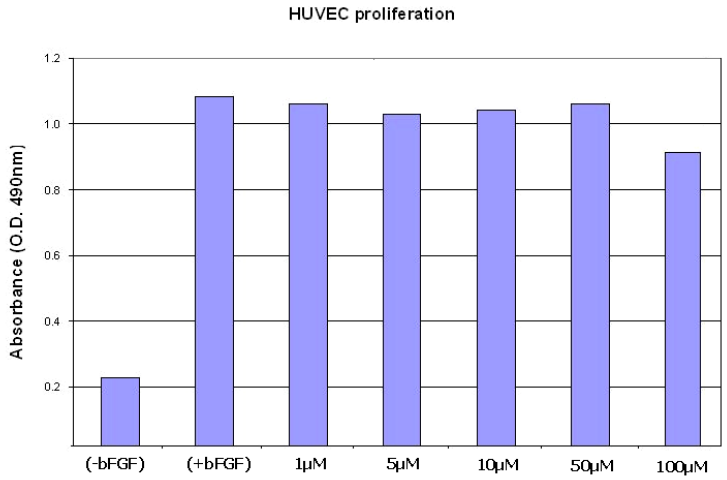

Fig. 5. Data showing MPC does not inhibit the proliferation of HUVE cells.

Intellectual Property Rights

A patent application has been filed with the US Patent and Trademark Office to protect the intellectual property rights described in this technical information brochure. A PCT application is approved by the World Intellectual Property Organization and now ready for national phase filing.

Summary

The findings described in this report indicate that menthol propyleneglycol carbonate (MPC), a GRAS food ingredient, is an effective nontoxic biochemical that may be used clinically as an anti-inflammatory and anti-androgenic agent. These observations open new market opportunities to use MPC in diverse medical conditions that involve angiogenesis and inflammation.