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GLYCERIDES OF ESSENTIAL  
POLYUNSATURATED FATTY ACIDS**(30) **Foreign Application Priority Data**

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**549/372**(21) Appl. No.: **11/885,254**(57) **ABSTRACT**(22) PCT Filed: **Mar. 2, 2006**(86) PCT No.: **PCT/GB06/00779**§ 371 (c)(1),  
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A method of treating a patient in need of therapy for a cytokine dysregulation comprising administering to that patient a therapeutically effective dose of a compound of general formula: (I) wherein  $R^1$  and  $R^2$  together form a group  $-(CH_2)_n-CR^4R^5-(CH_2)_m-$  wherein  $n$  and  $m$  are independently selected integers 0, 1 or 2 and  $R^4$  and  $R^5$  are independently selected from H,  $C_{1-18}$  alkyl,  $C_{1-18}$  alkoxy,  $C_{1-18}$  hydroxyalkyl,  $C_{2-18}$  alkenyl and  $C_{6-18}$  aryl or aralkyl and  $R^3$  is the fatty acyl group of an essential polyunsaturated fatty acid.

Fig. 1.

Peripheral Blood Mononuclear Cell Cytokine Production in Placebo and Oil Treated Multiple Sclerosis Patients at 18 Months

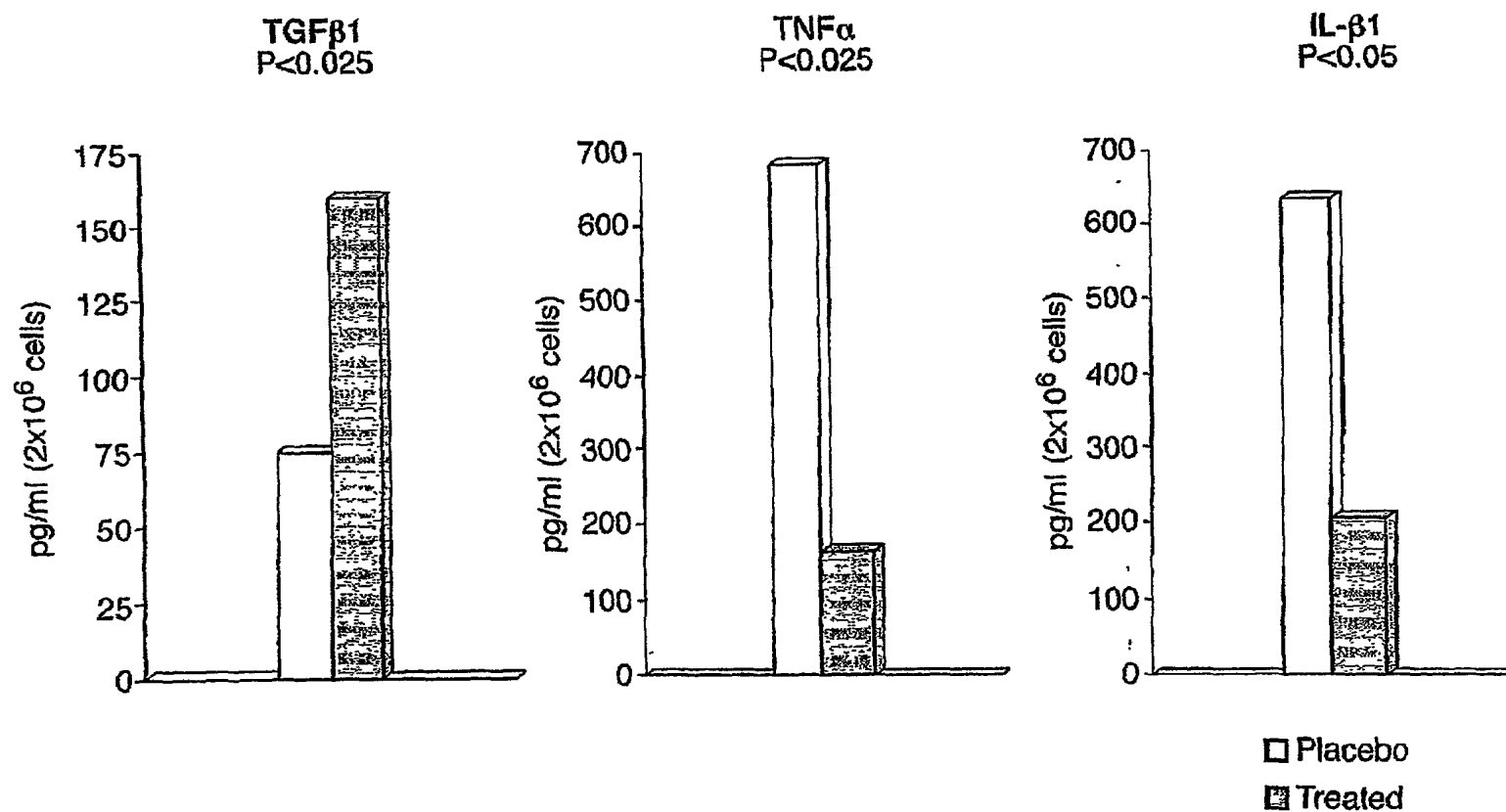


FIGURE2 and FIGURE 3

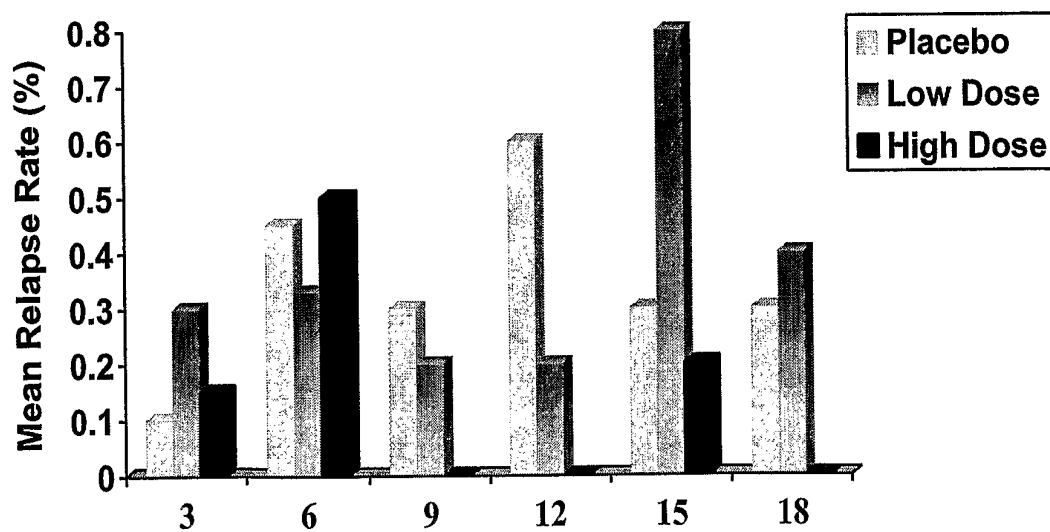
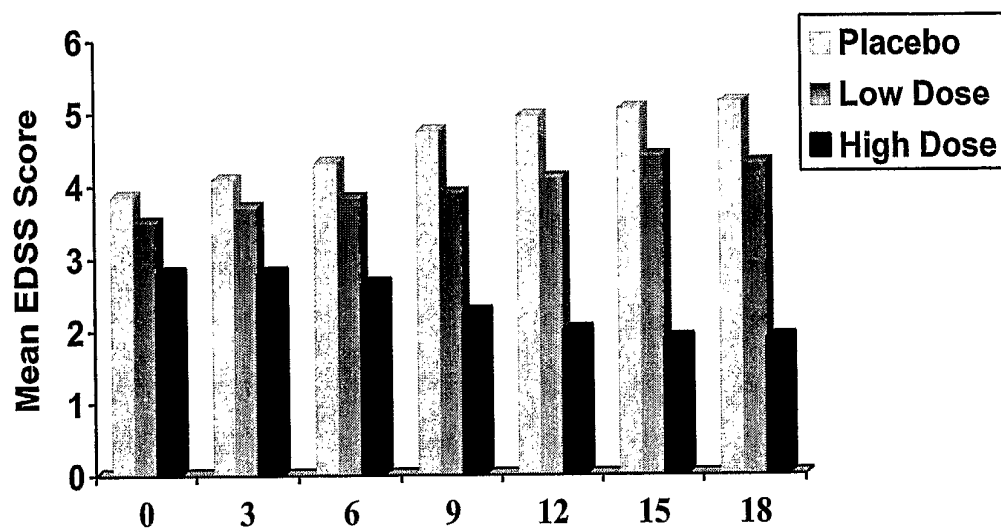
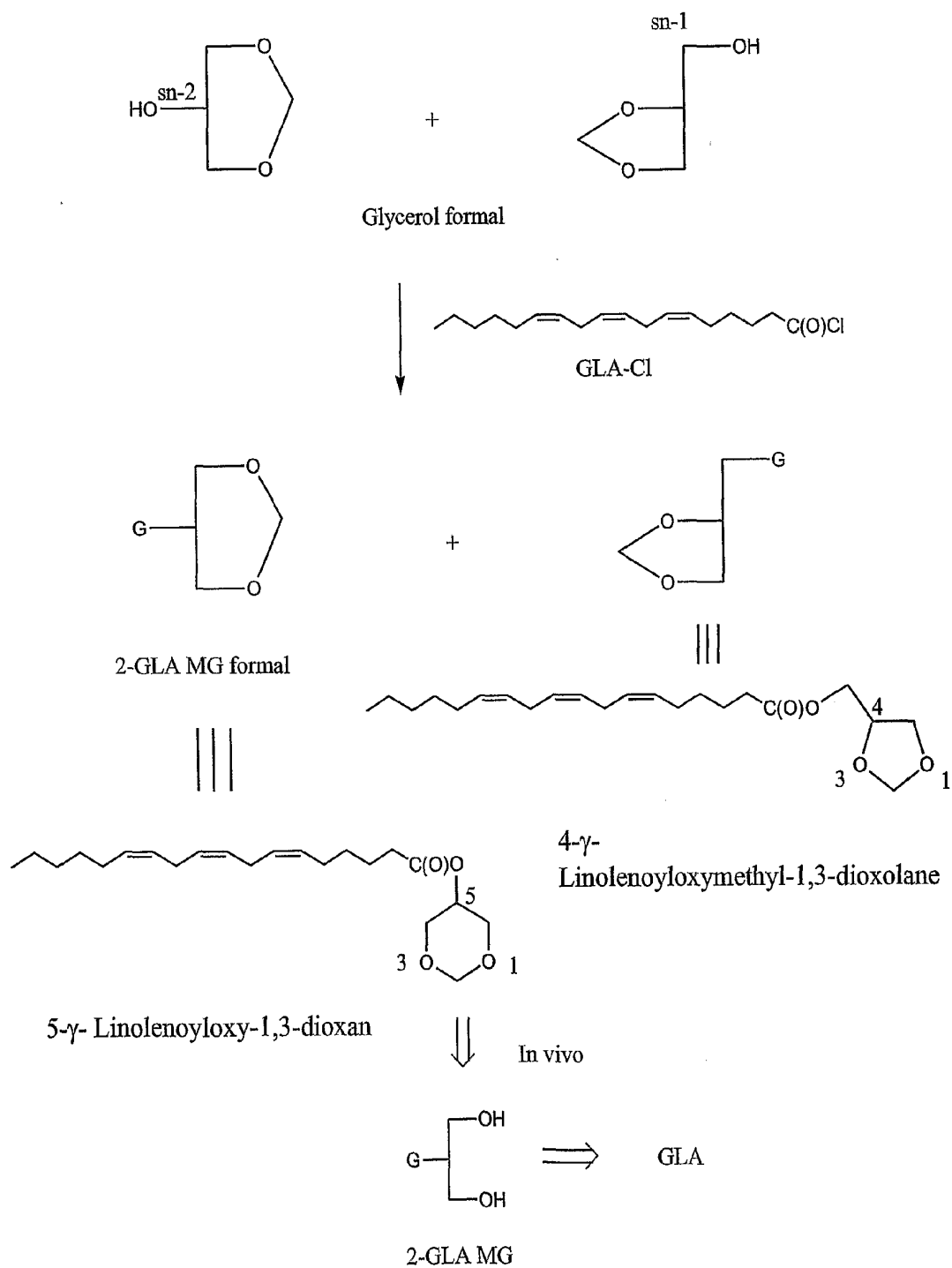


FIGURE 4

2- $\gamma$ - Linolenoyl glycerol formal(2-GLA MGF)



# **CYTOKINE MODULATORS USING CYCLIC GLYCERIDES OF ESSENTIAL POLYUNSATURATED FATTY ACIDS**

**[0001]** The present invention relates to a method for treating diseases and disorders in which cytokines are in state of imbalance or otherwise capable of modulation to provide therapeutic benefit. Particularly the invention provides a method of treatment of patients in need of therapy for disorders where the cytokines TGF- $\beta$ 1, TNF- $\alpha$ , IL-1 $\beta$ , IL4, IL5, IL6, IL8, IL10, IL13, and  $\gamma$ -IFN are dysregulated or capable of modulation to provide therapeutic benefit.

**[0002]** Particular diseases that are treatable by the present method are disorders such as abnormalities of the immune system, for example systemic lupus erythematosus (SLE), allergy, asthma, crohn's disease and rheumatoid arthritis, but particularly multiple sclerosis, and also neurodegenerative diseases such as sequelae of stroke, head trauma, bleeds and the chronic abnormalities of Alzheimer's and Parkinson's disease. Further disorders that can be pretreated both prophylactically and therapeutically are coronary heart disease (CHD) abnormalities of pre-mature infants and sepsis.

**[0003]** The inventor's copending patent application WO2004/100943 and WO2005/018632, incorporated herein by reference, relate to the use of synthetic, plant and fungal oils for the treatment of neurodegenerative diseases, particularly multiple sclerosis, stroke, head trauma, Alzheimer's and Parkinson's disease. WO2004/100943 relates to oils characterised by having at high percentages of the essential fatty acid  $\gamma$ -linolenic acid (GLA) at the sn-2 position of their lipids, typically being over 40% of the sn-2 fatty acid total of the oil. WO2005/018632 relates to structured lipids having an sn-2 fatty acid residue selected from  $\gamma$ -linolenic acid (GLA), dihomo- $\gamma$ -linolenic acid (DHGLA) and arachidonic acid (AA).

**[0004]** It is well reported in the literature that essential fatty acids (EFAs) of the n-3 and n-6 unsaturation pattern have beneficial effect in a wide variety of human physiological disorders, including autoimmune disease (WO 02/02105). Harbige (1998) Proc. Nut. Soc. 57, 555-562 reviewed the supplementation of diet with n-3 and n-6 acids in autoimmune disease states, and particularly noted evidence of benefit of  $\gamma$ -linolenic (GLA) and/or linoleic acid (LA) rich oils.

**[0005]** Cytokines are implicated in the pathogenesis of MS, with many studies showing an increase in myelinotoxic inflammatory cytokines (TNF- $\alpha$ , IL-1 $\beta$  and IFN- $\gamma$ ) coinciding with the relapse phase of the disease. Conversely, levels of the anti-inflammatory and immunosuppressive cytokine transforming growth factor-beta1 (TGF- $\beta$ 1) appear to be reduced during a phase of relapse and increase as the patient enters remission. Thus the balance between biologically active TGF- $\beta$ 1 and the pro-inflammatory TNF- $\alpha$ , IL-1 $\beta$  and IFN- $\gamma$  appears to be dysregulated during MS relapse-remission.

**[0006]** During natural recovery phase from EAE, TGF- $\beta$ 1-secreting T-cells inhibit EAE effector cells, TGF- $\beta$ 1 is expressed in the CNS and, in oral-tolerance-induced protection in EAE, TGF- $\beta$  and PGE<sub>2</sub> are expressed in the brain (Karpus & Swanborg (1991); Khoury et al (1992)). Harbige (1998) concluded that dietary  $\gamma$ -linolenic acid effects on EAE are mediated through Th<sub>3</sub>-like mechanisms involving TGF- $\beta$ 1 and possibly through superoxide dismutase antioxidant activity.

**[0007]** Borage oil (typically 20% to 23%  $\gamma$ -linolenic acid and 34 to 40% linoleic acid per 100% fatty acid content) and *Mucor javanicus* fungal oil (see FIG. 1) have been shown to be effective in the EAE animal model used to identify MS candidates, whilst never having been shown to be significantly effective in the human disease. High levels of linoleic rich oil containing low levels of  $\gamma$ -linolenic acid (EPO: linoleic acid: $\gamma$ -linolenic acid 7:1) partially suppressed the incidence and severity of EAE in rat (Mertin & Stackpoole, 1978) whereas the Bates' Naudicelle study referred to above led to worsening of patients. In spite of the use of Borage oil and other GLA/LA containing oils such as Evening Primrose oil by multiple sclerosis sufferers over the past 30 years or so, the vast majority of patients fail to recover from the disease, showing no significant improvement, with the underlying disease continuing to progress to death.

**[0008]** It has been suggested to use, inter alia,  $\gamma$ -linolenic acid and linoleic acid rich Borage oil as a means to provide immuno-suppression in multiple sclerosis (U.S. Pat. No. 4,058,594). Critically, the dose suggested is 2.4 grams of oil per day and no actual evidence of efficacy is provided. This is much lower than the low 5 g/day dose found to be ineffective in vivo in man in the WO2004/100943 study.

**[0009]** Other more dramatic immunosuppressant treatments, including T cell depleters and modulators such as cyclophosphamide, are also shown to be effective in the EAE model, but where these are employed in the human multiple sclerosis disease symptoms improve, but the underlying disease continues to progress. T-cells indeed produce beneficial cytokines, such as TGF- $\beta$ 1, as well as deleterious ones in man. David Baker of Institute of Neurology, UK summed up the disparity between what is effective in the EAE and in MS with a paper entitled 'Everything stops EAE, nothing stops MS' at the 10 May 2004 UK MS Frontiers meeting of the UK MS Society.

**[0010]** In the WO2004/100943 study the present inventors Harbige and Leach, with coinventor Sharief, surprisingly determined that with compliance to a 'high dose' treatment with triglyceride oil containing high levels of sn-2  $\gamma$ -linolenic acid (>40% of residues at the sn-2 being of  $\gamma$ -linolenic acid) with suitable accompanying fatty acid content, remarkable levels of improvement in almost all symptoms of MS can be achieved, way surpassing that provided by the current gold standard treatment. Such success is particularly surprising in the light of the prior use of other  $\gamma$ -linolenic acid containing preparations without success, such as the Naudicelle study.

**[0011]** The WO2004/100943 study shows that over an 18-month period, patients taking high dose (15 g/day) selected high sn-2  $\gamma$ -linolenic acid borage oil showed significant (p<0.001) and marked improvements in EDSS score, a reduced rate of relapse, symptomatic relief of muscle spasticity and painful sensory symptoms, and improved objective measures of cognitive functions. Low doses of 5 g/day of this borage oil were without effect.

**[0012]** Patients taking the highest dose of this borage oil maintained their level of peripheral blood mononuclear cell production (PBMC) of TGF- $\beta$ 1 during the trial period, their pro-inflammatory cytokines TNF- $\alpha$  and IL-1 $\beta$  were significantly and markedly (<70%) reduced and they either maintained or increased the PBMC membrane long chain omega-6 fatty acids dihomo- $\gamma$ -linolenic acid (DHGLA) and arachidonic acid (AA) in contrast to patients taking placebo who demonstrated loss of these fatty acids over the course of the trial period.

**[0013]** Thus whilst immuno-suppression would be expected to reduce increase of active lesioning and neurodegeneration, the high sn-2 GLA oil treatment apparently targeted maintenance and/or increase of key membrane lipid components that are otherwise specifically lost in MS, being consistent with a correction of a metabolic defect not otherwise effectively treated by current therapies. The fact that the low dose (5 grams/day) had no effect on this supports such determination.

**[0014]** The present inventors now set out, in view of the results obtained with high sn-2- $\gamma$ -linolenic acid Borage Oil, to demonstrate that it is indeed the presence of an sn-2- $\gamma$ -linolenic acid, dihomo- $\gamma$ -linolenic acid or arachidonic acid residue in a monoglyceride, particularly an sn-2 monoglyceride, or a metabolic precursors thereof, that gives it efficacy in treating cytokine dysregulation. Noting that the triglycerides themselves are of nearly three times the weight, and thus dose, of monoglyceride counterparts, they have determined that it is possible to administer essential fatty acids of the n-3, n-6 and n-9 type, particularly the n-6 type, as metabolic precursors or analogues of sn-2 monoglycerides and still obtain beneficial cytokine changes. Particularly these compounds are believed to be more stable than the equally less bulky monoglycerides.

**[0015]** It has been reported that 2-arachidonyl glycerol has activity in decreasing TNF- $\alpha$  and reactive oxygen species induced disease (see WO 01/97793), however, the corresponding  $\gamma$ -linolenoyl and palmitoyl compounds are said to be inactive. An sn-2-arachidonyl structured lipid has further been reported to be active in 'brain hypofunction' such as in mild cognitive impairment (see EP 1419768).

**[0016]** The dose advantages of use of monoglycerides over triglycerides may be offset in part by possible increased instability of certain forms as compared with the sn-1, sn-3 saturated acyl group sn-2 EFA triglyceride exemplified in PCT/GB2004/003524. Such instability may be due eg. to transesterification and oxidation. This issue may be addressed by producing the monoglyceride in a more stable form, eg a solid or semi solid rather than a liquid oil.

**[0017]** The present inventors have now further provided compounds, known and novel, which may be metabolic precursors of the monoglyceride as claimed below that are either metabolised to give that form in vivo, or are directly active, but which resist transesterification and other degradations in storage.

**[0018]** Preliminary studies by the inventors have shown that the compounds selected for the use of the present invention are more resistant to degradation by at least some mammalian lipases than the previously used triglyceride based Borage, Evening Primrose and structured lipids and more resistant to rearrangement than monoglycerides.

**[0019]** These compounds are particularly cyclic glycerols bearing essential fatty acids, particularly n-3, n-6 and n-9, but particularly n-6 fatty acids, in a position equivalent to the sn-2 position on the glycerol. The inventors have determined that these compounds are capable of regulating cytokines in a favourable manner to patient health based upon their previous clinical studies with Borage oil and the structured lipid in vivo and in vitro work.

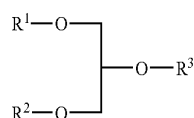
**[0020]** Cyclic glycerols are known as a class eg see. Chem Abstracts no 65:8747b and have been used as intermediates in the synthesis of monoacylglycerides eg see Chemical & Pharmaceutical Bulletin (2000), 48(7) 903-907. Recently US2005/0020679 has proposed that all members of this class

bearing a polyunsaturated fatty acyl group, particularly an arachidonyl group, have been proposed as having utility as anandamide transport inhibitors having pharmacological use in treatment of pain, peripheral pain, glaucoma, epilepsy, nausea, AIDS wasting, cancer, neurodegeneration, Multiple Sclerosis, Parkinson's Disease, Huntington's Chorea and Alzheimer's disease, enhancement of appetite, reduction of fertility, Tourettes and other motor function disorders, for neuroprotection, peripheral vasodilation and suppress memory.

**[0021]** This document and its predecessor WO99/64389 (directed to analgesia) provide no teaching of the particular compounds of the present invention and significantly teaches that a therapeutically effective amount of cyclic glycerol is 10 mg/day to 1000 mg/day. The present inventors studies on relative effect in animals and on cells has led them to determine that such a dose will be ineffective in the cytokine regulating indication now disclosed. The effective dose in man is expected to be above 1000 mg/day, particularly between 1 g to 10 g per day. More preferably being 2 to 6 g/day, still more preferably 2 to 4 g/day.

**[0022]** Without the cytokine regulation such dosages provide, treatment of diseases such as demyelinating diseases Multiple Sclerosis and Alzheimer's, Parkinson's, Huntington's Chorea and trauma and stroke with a view to halting ongoing immune cell and cytokine destruction of tissues cannot be achieved. Mere enhancement of natural anandamide levels by blocking reuptake cannot provide relief where natural anandamide is not present in effective amount, eg. in T-cells and around CNS lesions.

**[0023]** In a first aspect the present invention provides a method of treating a patient in need modulation of cytokines, particularly TGF- $\beta$ 1, TNF- $\alpha$ , IL-1 $\beta$ , IL4, IL5, IL6, IL8, IL10, IL13 and/or  $\gamma$ -IFN, comprising administering to that patient a therapeutically effective dose of a compound of general formula I



**[0024]** wherein R<sup>1</sup> and R<sup>2</sup> together form a group



**[0025]** wherein n and m are independently selected integers 0, 1 or 2

**[0026]** and R<sup>4</sup> and R<sup>5</sup> are independently selected from H, C<sub>1-18</sub> alkyl, C<sub>1-18</sub> alkoxy, C<sub>1-18</sub> hydroxyalkyl, C<sub>2-18</sub> alkenyl and C<sub>6-18</sub> aryl or aralkyl

**[0027]** and R<sup>3</sup> is the a fatty acyl group of an essential polyunsaturated fatty acid.

**[0028]** Preferred polyunsaturated fatty acyl groups are those of n-3, n-6 and n-9 fatty acids, more preferably n-3 and n-6 fatty acids, still more preferably being  $\gamma$ -linolenoyl,  $\gamma$ -dihomolinolenoyl and arachidonoyl.

**[0029]** Preferably R<sup>1</sup> and R<sup>2</sup> are selected from H or a group —CR<sup>4</sup>R<sup>5</sup> where R<sup>4</sup> and R<sup>5</sup> are independently selected from H and C<sub>1-6</sub> alkyl. Most advantageously R<sup>1</sup> and R<sup>2</sup> form a group —CH<sub>2</sub>— or —CH<sub>2</sub>CH<sub>2</sub>—.

**[0030]** Particularly preferred are compounds that are 5-essential fatty acyloxy-1,3-dioxans, more particularly n-3-fatty acyloxy or n-6 fatty acyloxy-1,3-dioxans, most preferably

5- $\gamma$ -linolenoyloxy, 5-dihomo- $\gamma$ -linolenoyloxy and 5-arachidonoyloxy-1,3-dioxans. Most preferable are the non-inflammatory 5- $\gamma$ -linolenoyloxy and 5-dihomo- $\gamma$ -linolenoyloxy-1,3-dioxans.

[0031] Particularly treated are dysregulated cytokine abnormalities of the immune system, for example diseases such as systemic lupus erythematosus (SLE), allergy, asthma, crohn's disease and rheumatoid arthritis, but particularly multiple sclerosis, and also neurodegenerative diseases such as sequelae of stroke, head trauma, bleeds and the chronic abnormalities of Alzheimer's and Parkinson's disease. Further disorders that can be pretreated both prophylactically and therapeutically are coronary heart disease (CHD) abnormalities of premature infants and sepsis.

[0032] Particularly advantageously treated neurodegenerative diseases are those involving demyelination. The present method is specifically addressed at arresting underlying neurodegeneration and restoring neuronal function. Particularly the method preferably normalises neuronal membrane composition, and restores healthy PBMC stimulated or spontaneously released TGF- $\beta$ 1/TNF $\alpha$  ratios and the ratios of TGF- $\beta$ 1 with other PBMC released cytokines. Most advantageously the method arrests neurodegeneration in multiple sclerosis of all types but particularly relapsing remitting, primary progressive and chronic progressive MS and the restoration, in part or completely, of neuronal function such as measured, eg. By MRI or CAT scan or by EDSS score. Such method may also be used in treatment of cerebral impairment after stroke, head trauma and intracranial bleeding where there is demyelination or neuronal damage. Further application is provided in treating other chronic demyelination such as in Alzheimer's and Parkinson's disease.

[0033] Preferably the compound of the present invention is administered for a duration and at a dose sufficient to maintain or elevate TGF- $\beta$ 1 levels in the patient to therapeutic levels. By therapeutic levels is meant levels at least consistent with healthy subjects. Preferably the dose is such as to produce a TGF- $\beta$ 1/TNF- $\alpha$  ratio spontaneously released from peripheral blood mononuclear cells (PBMCs) isolated from blood of a patient, after 18 months of daily dosing, of 0.4 to 3.0, at least 0.5, more preferably at least 0.75 and most preferably at least 1. Preferably the dose is such as to produce a TGF- $\beta$ 1/IL-1 $\beta$  ratio in blood of a patient, after 18 months of daily dosing, of at least 0.5, more preferably at least 0.75 and most preferably at least 1. Preferably said levels are produced after 12 months and more preferably after 6 months.

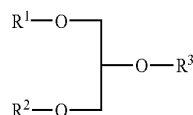
[0034] The present invention further provides a method of treating a patient in need of remyelination in a demyelinating disease comprising administering to that patient a therapeutically effective amount of a compound of formula I wherein R<sup>3</sup> is selected from  $\gamma$ -linolenoyl,  $\gamma$ -dihomolinolenoyl and arachidonoyl; most preferably  $\gamma$ -linolenoyl and  $\gamma$ -dihomolinolenoyl.

[0035] For all the methods of the invention, the amount of compound administered daily will be between 0.5 and 30 grams, orally dosed, still more preferably between 0.75 and 20 grams and most preferably between 1 and 18 grams, typically 2 to 5 grams. This dose may be given as one single dose or in two or more doses together totally this amount per day.

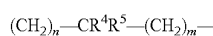
[0036] Where the sn-2 moiety is that of a  $\gamma$ -linolenic acid residue, the dose may be toward the higher end of these ranges, but is preferably between 1 and 10 grams/day, more preferably 2 to 6 grams/day. Where the sn-2 moiety is that of a dihomom- $\gamma$ -linolenic acid residue, the dose may be less,

whilst where the sn-2 moiety is that of an arachidonic acid residue, efficacy is higher, but dosing should be more cautious, due to possibilities of unwanted side effects at higher levels and the pro-inflammatory nature of this PUFA.

A second aspect of the present invention provides novel compound of formula I



[0037] wherein R<sup>1</sup> and R<sup>2</sup> together form a group



[0038] wherein n and m are independently selected integers 0, 1 or 2

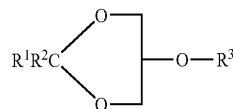
[0039] and R<sup>4</sup> and R<sup>5</sup> are independently selected from H, C<sub>1-18</sub> alkyl, C<sub>1-18</sub> alkoxy, C<sub>1-18</sub> hydroxyalkyl, C<sub>2-18</sub> alkenyl and C<sub>6-18</sub> aralkyl

[0040] and R<sup>3</sup> is a fatty acyl group of an essential polyunsaturated fatty acid.

[0041] Preferred polyunsaturated fatty acyl groups are those of n-3, n-6 and n-9 fatty acids, more preferably n-3 and n-6 fatty acids, still more preferably being  $\gamma$ -linolenoyl,  $\gamma$ -dihomolinolenoyl and arachidonoyl.

[0042] Preferably R<sup>1</sup> and R<sup>2</sup> form a group —CR<sup>4</sup>R<sup>5</sup> where R<sup>4</sup> and R<sup>5</sup> are independently selected from H and C<sub>1-6</sub> alkyl. Most advantageously R<sup>1</sup> and R<sup>2</sup> form a group —CH<sub>2</sub>— or —CH<sub>2</sub>CH<sub>2</sub>—.

[0043] Thus compounds of formula IIa are preferred



[0044] wherein R<sup>1</sup> and R<sup>2</sup> are independently selected from hydrogen and C<sub>1-6</sub> alkyl.

[0045] Particularly preferred are compounds that are 5-acyloxy-1,3-dioxans, more particularly n-3-fatty acyl or n-6 fatty acyloxy-1,3-dioxans, most preferably 5- $\gamma$ -linolenoyloxy, 5-dihomo- $\gamma$ -linolenoyloxy and 5-arachidonoyloxy-1,3-dioxans.

[0046] A third aspect of the present invention provides a method for synthesis of a compound of general formula II comprising reaction a 5-hydroxy-1,3-dioxan with an essential fatty acid or an essential fatty acid halide.

[0047] A fourth aspect of the present invention provides compositions for use in the method of the present invention comprising the compounds of formula I together with a pharmaceutically or nutraceutically acceptable carrier, coating, capsule, diluent and/or preservative. The compounds for use in the present invention may be administered by any of the conventional vehicles known in pharmacy. Most conveniently they are administered as neat oils or in admixture with foodstuffs, in the form of capsules containing such oils, or in enterically coated forms. Other forms will occur to those skilled in the art but Remington Pharmaceutical Sciences 19<sup>th</sup> Edition

[0048] By preservative is meant an antioxidant or inhibitor of transesterification. It is particularly preferred that the composition does not include Vitamin E, or includes only levels of Vitamin E that are 0.05 mg/g or less, eg 0.005 to 0.05 mg/g.

**[0049]** A fifth aspect of the present invention provides a pharmaceutical composition for regulating the immune system, particularly by modulating cytokines TGF- $\beta$ 1, TNF- $\alpha$ , IL4, IL5, IL6, IL8, IL10, IL13, and/or  $\gamma$ -IFN comprising a compound of general formula I as defined for the method of treatment of the invention.

**[0050]** A sixth aspect of the present invention provides use of the compounds of formula I, formula II and formula IIa as described above for the manufacture of a medicament for the treatment of cytokine dysregulation and neurodegenerative diseases as set out for the method of the invention. Particularly preferred medicaments are for the arresting and reversing of neurodegeneration in multiple sclerosis of all types but particularly relapsing remitting, primary progressive and chronic progressive and the restoration, in part or completely, of neuronal integrity function such as measured, eg. By MRI or CAT scan or by EDSS score. Other TGF- $\beta$ 1 responsive diseases may be treated as set out previously. Particularly treated is demyelination.

**[0051]** It will be realised by those skilled in the art that other beneficial agents may be combined with the compounds for use in the present invention or otherwise form part of a treatment regime. These might be ion channel blockers, eg. sodium channel blockers, interferons ( $\alpha$ ,  $\beta$ , or  $\gamma$ ), T-cell depleters, steroids or other palliative agents. It will further be realised that where the immune and inflammatory responses are being modulated, such combinations will need to be made carefully, given the complex nature of these systems. However, given the potential for delayed response to the present compounds, shorter acting agents might be beneficial in the first months of treatment before the cytokine levels are normalised, as long as the additional treatment does not impede this normalization process.

**[0052]** The synthesis of compounds and compositions for use in the present invention is described below together with synthesis of comparative examples. Whilst the Examples exemplify compounds where R<sup>3</sup> is  $\gamma$ -linolenoyl, the dihomomethyl- $\gamma$ -linolenoyl and arachidonoyl and n-3 and n-9 analogues of these are readily synthesised through analogous starting materials.

**[0053]** The present invention will now be described by way of Example only by reference to the following non-limiting Tables, Examples and Figures. Further embodiments falling within the scope of the invention will occur to those skilled in the art in the light of these.

#### Tables

#### FIGURES

**[0054]** FIG. 1: Shows spontaneous peripheral blood mononuclear cell cytokine production in placebo and high sn-2  $\gamma$ -linolenic acid, WO2004/100943 trial oil treated human MS patients at 18 months. The left hand column in each case is placebo: right treated.

**[0055]** FIG. 2: Shows the effect of placebo and low dose (5 g/day) high sn-2 GLA Borage oil on human MS patient EDSS score as compared to high dose (15 g/day) displayed as a histogram with months treatment on the x axis.

**[0056]** FIG. 3: Shows the effect of placebo, low dose and high dose high sn-2 GLA Borage oil on human MS patient Mean Relapse rate (%) as histogram with months on x axis.

**[0057]** FIG. 4: Shows synthesis of 2-GLA glycerol formal (aka: 5- $\gamma$ -linolenyloxy)-1,3-dioxan and 5-(1,3-dioxanyl)methyl- $\gamma$ -linolenate)

#### EXAMPLES

##### Background

**[0058]** High sn-2 Borage Oil (WO2004/100943) Trial.

##### Isolation and Culture of PBMC

**[0059]** Heparinised whole blood was diluted with an equal volume of Hanks' balanced salt solution (Sigma, UK) and the

resulting diluted blood layered onto Lymphoprep (Nycomed, Oslo, Norway). Following density centrifugation at 800 g for 30 minutes the PBMC were removed from the interface and diluted in Hanks' solution. The cells were then washed twice by centrifugation for 10 minutes at 250 g. The resulting final pellet was then resuspended in culture medium consisting of RPMI-1640 medium (Sigma, UK) supplemented with 2 mM L-glutamine, 100 U penicillin and 100  $\mu$ g streptomycin (Sigma, UK) and 10% autologous plasma.  $2 \times 10^6$  per ml PBMC, >95% viable as judged by trypan blue exclusion, were added to tissue culture tubes (Bibby Sterilin Ltd, Stone, UK) and incubated for 24 h at 37° C. with 5% CO<sub>2</sub>. The concentration of antigen, cell density and time of culture were all determined in previous kinetic experiments to determine maximum cytokine production (data not shown). Routine cytopsin preparations were also prepared for subsequent differential counts. Following incubation the cells were removed from culture by centrifugation at 250 g for 10 minutes, the resulting supernatants were then removed, aliquoted and stored at -70° C.

##### Preparation of Plasma Samples

**[0060]** 10 ml of heparinised blood was spun at 250 g for 10 minutes. The resulting plasma layer was then removed, aliquoted and stored at -70° C.

##### Detection of Pro-Inflammatory Cytokines

**[0061]** TNF- $\alpha$ , IL-1 $\beta$  and IFN- $\gamma$  in cell culture supernatants and plasma were detected using commercially available paired antibodies enabling cytokine detection in an ELISA format (R&D systems Ltd, Abingdon, UK). The sensitivities for the TNF- $\alpha$  and IFN- $\gamma$  ELISAs were 15.6-1000 pg/ml and 3.9-250 pg/ml for IL-1 $\beta$ .

##### Detection of Biologically Active TGF- $\beta$ 1

**[0062]** Biologically active TGF- $\beta$ 1 in cell culture supernatants and plasma were detected using the commercially available E<sub>max</sub> ELISA system with a sensitivity of 15.6-1000 pg/ml (Promega, Southampton, UK).

##### Statistical Analysis

**[0063]** Differences in cytokine production were compared using Student's t-test and Mann-Whitney U-test and were considered significant when p values were less than 0.05.

##### Results

See FIG. 1

##### Experimental Procedure

**[0064]** The proton-decoupled <sup>13</sup>C NMR spectra with suppressed NOE were collected at 21° C. in a 5-mm broadband probe on a Joel 500 MHz spectrometer operating at 125.728 MHz. Waltz decoupling was the chosen mode of decoupling and was gated on only during the 14.89s acquisition time. The relaxation delay was set at 30 secs and the pulse angle was 90°. The spectral window used was ca.35 ppm (from 173.5 to 172.6 ppm) with a 170 ppm offset. The spectra were internally referenced to CDCl<sub>3</sub> at 77.0 ppm. Typically, the approximate number of scans collected for adequate signal-to-noise ranged from 300 to 1200 scans depending on the concentration and purity of the sample. The total acquisition time for the experiments ranged between 2-8 h e.g 1272 scans; data points 65,536. Concentrated solutions up to 20%



w/v were employed when possible to reduce the acquisition time. The chemical shifts quoted vary with the concentration of the solution.

#### Synthesis of Compounds for Use in the Present Invention.

##### Example 1

1a) Preparation of 1,3-O-benzylidene glycerol 2-octa-6Z, 9Z, 12Z-trienoate. (Intermediate and Known Compound for Use in the Invention where R4 is H and R5 is Benzyl)

**[0065]** Oxaloyl chloride (7.8 ml, 11.3 g, 0.089 mol, 0.95 equivalents) was added over 2-3 minutes to a stirred solution of  $\gamma$ -linolenic acid (GLA95, 16.7 g, 0.060 mol, 0.64 equivalents-Scotia) in dichloromethane (100 ml) under  $N_2$ . The mixture was stirred overnight at room temperature and then concentrated in vacuo to give a tan oil. This crude  $\gamma$ -linolenoyl chloride was added over ca 10 minutes to a stirred solution of 1,3-O-benzylidene glycerol (13.0 g, 0.094 mol, 1 equivalent), dry pyridine (30 ml, 29.3 g, 0.37 mol, 4 equivalents) and dichloromethane (DCM, 120 ml) at 5° C. and the mixture then stirred at room temperature for 2 hours. The reaction mixture was filtered and then the filtrate washed with DCM. The combined filtrate and washings were then washed with water (2x20 ml) and the DCM extract dried over  $MgSO_4$  and concentrated in vacuo to give a crude product as a tan oil (purity >90% by HPLC). The oil was purified by column chromatography on silica gel (300 g). Elution with DCM gave the product as a yellow oil (19.2 g (73%), 96.3% purity by HPLC).

##### Example 2

2-GLA glycerol formal (aka: 5- $\gamma$ -linolenoyloxy)-1,3-dioxan and 5-(1,3-dioxanyl)-methyl- $\gamma$ -linolenate)

**[0066]** For the purposes of this exemplification, the synthesis of this ester was carried out by acylation of the commercially available glycerol formal mixture using  $\gamma$ -linolenoyl chloride. By this method a mixture of two products is formed and these were separable by column chromatography. The undesired by-product is eluted off the column first; further elution gives the desired acetal-ester. It is a yellow oil at room temperature and appears to have stability properties similar to GLA, stable in air at room temperature for short periods (days) but is best stored long term in a cool place under nitrogen.

#### Experimental

**[0067]** Oxaloyl chloride (2.6 ml, 3.78 g, 30 mmol, 1.5 equiv) was added to a solution of  $\gamma$ -linolenic acid (GLA, 5.56 g, 20 mmol, 1.0 equiv) in dichloromethane (DCM, 40 ml). The resulting solution was stirred under  $N_2$  at room temperature overnight and then concentrated in vacuo. The residual oily  $\gamma$ -linolenoyl chloride was added dropwise over 10 min to a stirred solution of glycerol formal (2.50 g, 24 mmol, 1.2 equiv) in DCM (40 ml) containing pyridine (10 ml, 9.78 g, 0.12 mol, 6 equiv) at 5° C. The reaction mixture was stirred at room temperature for 2 h, the precipitated pyridine hydrochloride filtered off, and the filtrate washed with water (2x). After drying over  $MgSO_4$  the solvent was removed in vacuo to give a light tan oil (6.5 g). This material was chromatographed on silica (60 g). Elution with hexane-ether (94:6) gave 5.2 g of an oil consisting of two components (TLC, HPLC). These were separated on a second silica column (60 g). Elution with hexane-ether (98:2 then 95:5) gave 4-( $\gamma$ -linolenoyloxymethyl)-1,3-dioxolane as a yellow oil (1.2 g, 98% by HPLC).  $\delta_H$  (500 MHz,  $CDCl_3$ ) 0.89 (3H, t, J=7.0 Hz,

$CH_3$ ), 1.24-1.45 (8H, complex m,  $4 \times CH_2$ ), 1.65 (2H, p, J=7.5 Hz,  $CH_2-C-CO$ ), 2.08 (4H, m,  $2 \times CH_2C=C$ ), 2.35 (2H, t, J=7.5 Hz,  $CH_2CO$ ), 2.80 (4H, t, J=6.0 Hz,  $2 \times C=CCH_2C=C$ ), 3.67 (1H, m,  $OCH_4H_B$ ), 3.97 (1H, m,  $OCH_4H_B$ ), 4.14 (2H, m,  $OCH_4H_B$ ), 4.26 (1H, p, J=3.5 Hz, CHO), 4.89 and 5.02 (2H, 2xs,  $OCH_2O$ ), 5.36 (6H, m,  $3 \times CH=CH$ ).  $\delta_C$  (126.8 MHz,  $CDCl_3$ ) 14.09 ( $CH_3$ ), 22.60, 24.51, 25.65, 26.85, 27.23, 29.16, 29.34, 31.53, 33.97, 63.93 ( $CH_2O$ ), 66.72 ( $CH_2O$ ), 73.31 (CHO), 95.44 (OCO), [127.60, 128.04, 128.32, 128.41, 129.50, 130.41, olefinic C], 173.26 (carbonyl).

**[0068]** Further elution gave 5-( $\gamma$ -linolenoyloxy)-1,3-dioxan as a yellow oil (1.6 g, 97.8%) by HPLC).  $\delta_H$  (500 MHz,  $CDCl_3$ ) 0.89 (3H, t, J=7.0 Hz,  $CH_3$ ), 1.24-1.46 (8H, complex m,  $4 \times CH_2$ ), 1.67 (2H, p, J=7.5 Hz,  $CH_2-C-CO$ ), 2.05 (4H, m,  $2 \times CH_2C=C$ ), 2.40 (2H, t, J=7.5 Hz,  $CH_2CO$ ), 2.81 (4H, t, J=6.0 Hz,  $2 \times C=CCH_2C=C$ ), 3.91 (2H, m,  $OCH_2$ ), 3.99 (2H, m,  $OCH_2$ ), 4.73 (1H, p, J=3.5 Hz, CHO), 4.80 (1H, d, J=6.0 Hz,  $OCH_4H_BO$ ), 4.93 (1H, d, J=6.0 Hz,  $OCH_4H_BO$ ), 5.37 (6H, m,  $3 \times CH=CH$ ).  $\delta_C$  (126.8 MHz,  $CDCl_3$ ) 14.08 ( $CH_3$ ), 22.59, 24.53, 25.65, 26.86, 27.22, 29.06, 29.34, 31.52, 34.12, 65.54 (CHO), 68.55 ( $CH_2O$ ), 93.66 (OCO), [127.60, 128.05, 128.32, 128.42, 129.51, 130.42, olefinic C], 173.12 (carbonyl). Some fractions containing both compounds were obtained during the chromatography and these could be recycled if necessary to give more material. The reaction scheme for this synthesis is shown in the figures below.

##### Example 3

Production of Monoglyceride Enriched Compositions from Synthetic CGC Structured-Lipid. Model Compound for Metabolite of Compounds of the Invention

**[0069]** 1. 2-GLA MG ( $\gamma$ -linolenic acid monoglyceride) from CGC (Glycerol 1,3-didecanoate-2- $\gamma$ -linolenate)

**[0070]** Lipase acrylic resin from *Candida antarctica* (Sigma, Novozyme, 0.1 g) was added to a solution of CGC (0.25 g) in ethanol (0.75 ml). The mixture was stirred at 3540° C. and monitored by HPLC. After 3 h the resin was removed by filtration and washed with ethanol. The filtrate and washings were concentrated in vacuo. Analysis of the residual oil by HPLC indicated the formation of two major products. These were separated by chromatography on silica-boric acid. Elution with dichloromethane (DCM) gave an oil (fraction A, 160 mg). Further elution with DCM-MeOH (9:1) gave an oil (fraction B, 80 mg). HPLC comparison with authentic materials indicated that B was the required product i.e. 2-GLA MG (8% rearranged 1-isomer also present). The main (>90%) component of fraction A was found (by HPLC comparison and NMR) to be ethyl decanoate. The minor component was found (by HPLC and NMR) to be ethyl  $\gamma$ -linolenate. These esters are expected to be formed under the reaction conditions from the corresponding acids (C and GLA) and ethanol.

#### Biological Studies.

##### Example 4

**[0071]** Solubilization of Sn-2 monoglyceride was performed using ethyl alcohol or DMSO for in vitro work on human peripheral blood mononuclear cells (PBMCs). A tendency to precipitate at acid pH may have been the cause of some animals regurgitating solid material after gavage suggesting that enterically coated formulation may be preferred. SJL mice were fed sn-2 GLA of Example 1 at three doses (50,

125 and 250  $\mu$ l) for seven days by gavage. Mice receiving higher doses were prone to regurgitation. After seven days animals were killed and the brain, liver and spleen were removed the liver and brain frozen at  $-70^{\circ}$  C. and mononuclear cells were isolated from spleens by sieving and density centrifugation on Lymphoprep (Sigma Chemical Co) and cultured at  $37^{\circ}$  C. in 5%  $\text{CO}_2$  atmosphere in 511 culture tubes at a cell density of  $1 \times 10^6$  cells/ml in RPMI 1640 medium in 5% foetal calf serum (FCS). Cells were cultured with and without 1  $\mu$ g/ml or 25  $\mu$ g/ml concanavalin A (Con A) for approximately 20 hours and the supernatants removed and stored at  $-70^{\circ}$  C. until required. Mouse TGF- $\beta$ 1 was measured in supernatants using a commercially available ELISA (Promega, Madison Wis.).

TABLE 1

Stimulated (Con A) and unstimulated TGF- $\beta$ 1 production pg/ml from spleen PBMCs in response to feeding of sn-2- $\gamma$ -linolenoyl-glycerol monoglyceride of Example 3			
Monoglyceride	Con A ( $\mu$ g/ml)		
	0	1	25
50 $\mu$ g	377	409	480
Control	209	228	393
Change %	80	79	42

[0072] This shows that the metabolite of the  $\gamma$ -linolenoyl compound of formula of Example 2 is active in raising cytokine TGF- $\beta$ 1 in the same manner as Borage oil of the trial. This is consistent with modulation of all the cytokines previously shown modulated in this matter.

### Example 5

#### In Vivo Activity of Monoglyceride Formal (Compound of Example 2)

[0073] C57/BL mice were dosed orally with 2 GLA MG formal at two doses of 50 and 150  $\mu$ l for eight days. The compound was supplied in 8 separate tubes to be stored at  $4^{\circ}$  C. prior to dosing but warmed to  $25^{\circ}$  C. before use. After eight days the animals were killed and the brain, liver and spleen were removed. Liver and brain were frozen at  $-70^{\circ}$  C. and mononuclear cells were isolated from spleens by sieving and density centrifugation on Lymphoprep (Sigma Chemical Co) and cultured at  $37^{\circ}$  C. in 5%  $\text{CO}_2$  atmosphere in 5 ml culture tubes at a cell density of  $2 \times 10^6$  cells/ml in RPMI 1640 medium in 5% fetal calf serum (FCS). Cells were cultured with and without 1  $\mu$ g/ml or 25  $\mu$ g/ml concanavalin A (Con A) for approximately 20 hrs and the supernatants removed and stored at  $-70^{\circ}$  C. until required. Mouse TGF- $\beta$ 1 was measured in supernatants using a commercially available ELISA system (Pomoga, Madison, Wis.).

#### Results:

[0074] Concanavalin A did not significantly stimulate TGF- $\beta$ 1 production in this study. Data were therefore combined for the purposes of statistical analysis ( $n=9$ /group). Data were analysed using Graphpad Instat.

TABLE 2

Spleen mononuclear cell TGF $\beta$ production (pg/ml)		
Controls	77.9 $\pm$ 4.1	
GLA 2-MG-F50 $\mu$ l	90.9 $\pm$ 5.2	P = 0.0702
GLA 2-MG-F 150 $\mu$ l	158.6 $\pm$ 7.5	P < 0.0001 $\uparrow$ 103%

[Data analysed using unpaired t-test; data are mean  $\pm$  SEM]

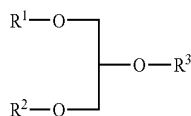
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1. A method of treating a patient in need of cytokine modulation comprising administering to that patient a therapeutically effective dose of a glyceride or compound of general formula I



wherein R<sup>1</sup> and R<sup>2</sup> together form a group



wherein n and m are independently selected integers 0, 1 or 2

and R<sup>4</sup> and R<sup>5</sup> are independently selected from H, C<sub>1-18</sub> alkyl, C<sub>1-18</sub> alkoxy, C<sub>1-18</sub> hydroxyalkyl, C<sub>2-18</sub> alkenyl and C<sub>6-18</sub> aryl or aralkyl

and R<sup>3</sup> is the a fatty acyl group of an essential polyunsaturated fatty acid.

2. A method as claimed in claim 1 wherein the polyunsaturated fatty acyl group is selected from those of n-3, n-6 and n-9 fatty acids, more preferably n-3 and n-6 fatty acids, still more preferably being  $\gamma$ -linolenoyl,  $\gamma$ -dihomolinenoyl and arachidonoyl.

3. A method as claimed in claim 1 wherein R<sup>1</sup> and R<sup>2</sup> form a group  $-\text{CR}^4\text{R}^5$  wherein R<sup>4</sup> and R<sup>5</sup> are independently selected from H and C<sub>1-6</sub> alkyl.

4. A method as claimed in claim 1 wherein R<sup>1</sup> and R<sup>2</sup> form a group  $-\text{CH}_2-$  or  $-\text{CH}_2\text{CH}_2-$ .

5. A method as claimed in claim 1 wherein the compound of formula I is a 5-essential fatty acyloxy-1,3-dioxans.

6. A method as claimed in claim 1 wherein the compound of claim 1 is an n-3 fatty acyloxy-1,3-dioxan or an n-6 fatty acyloxy-1,3-dioxans.

7. A method as claimed in claim 1 wherein the compound of formula I is selected from a 5- $\gamma$ -linolenyloxy-1,3-dioxans, 5-dihomo- $\gamma$ -linolenyloxy-1,3-dioxans and 5-arachidonoyl-1,3-dioxans.

8. A method as claimed in claim 1 wherein the patient is in need of therapy for cytokine dysregulation.

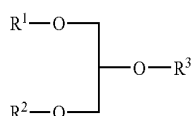
9. A method as claimed in claim 1 wherein the patient is in need of therapy to prevent demyelination in a neurodegenerative disease.

10. A method as claimed in claim 1 wherein the modulated cytokines are one or more of TGF- $\beta$ 1, TNF- $\alpha$ , IL-1, IL4, IL5, IL6, IL8, IL10, IL13, and  $\gamma$ -IFN.

11. A method as claimed in claim 1 wherein the treatment is for abnormalities of the immune system.

12. A method as claimed in claim 11 wherein the treatment is for a condition selected from the group consisting of systemic lupus erythematosus (SLE), allergy, asthma, crohn's disease and rheumatoid arthritis, multiple sclerosis, neurodegenerative diseases sequelae of stroke, head trauma, bleeds and the chronic abnormalities of Alzheimer's and Parkinson's disease, coronary heart disease (CHD) abnormalities of premature infants and sepsis.

13. A compound of formula I



wherein R<sup>1</sup> and R<sup>2</sup> together form a group



wherein n and m are independently selected integers 0, 1 or 2

and R<sup>4</sup> and R<sup>5</sup> are independently selected from H, C<sub>1-18</sub> alkyl, C<sub>1-18</sub> alkoxy, C<sub>1-18</sub> hydroxyalkyl, C<sub>2-18</sub> alkenyl and C<sub>6-18</sub> aralkyl

and R<sup>3</sup> is a fatty acyloxy group of an essential polyunsaturated fatty acid.

14. A compound as claimed in claim 13 wherein R<sup>3</sup> is a polyunsaturated fatty acyl groups of an n-3, n-6 and n-9 fatty acids, more preferably n-3 and n-6 fatty acids, still more preferably being  $\gamma$ -linolenoyl,  $\gamma$ -dihomolinenoyl and arachidonoyl.

15. A compound as claimed in claim 13 wherein R<sup>1</sup> and R<sup>2</sup> form a group  $-\text{CR}^4\text{R}^5$  where R<sup>4</sup> and R<sup>5</sup> are independently selected from H and C<sub>1-6</sub> alkyl.

16. A compound as claimed in claim 13, wherein R<sup>1</sup> and R<sup>2</sup> form a group  $-\text{CH}_2-$  or  $-\text{CH}_2\text{CH}_2-$ .

17. A compound as claimed in claim 13 being a 5-acyloxy-1,3-dioxan.

18. A compound as claimed in claim 13 being an n-3-fatty acyl or n-6 fatty acyloxy-1,3-dioxans.

19. A compound as claimed in claim 13 being a 5- $\gamma$ -linolenyloxy-1,3-dioxan, a 5-dihomo- $\gamma$ -linolenyloxy-1,3-dioxan or a 5-arachidonoyloxy-1,3-dioxan.

20. A compound as claimed in claim 13 for use in therapy.

21. A method for synthesis of a compound of general formula I comprising reacting a 5-hydroxy-1,3-dioxan with an essential fatty acid or an essential fatty acid halide.

22. A composition for use in the method of the present invention comprising a compound of formula I together with a pharmaceutically or nutraceutically acceptable carrier, coating, capsule, diluent and/or preservative.

23. A composition as claimed in claim 22 comprising a preservative which is an antioxidant or inhibitor of transesterification.

24. A composition as claimed in claim 23 comprising 0.05 mg/g or less of Vitamin E.

25. A pharmaceutical composition for regulating the immune system comprising a compound of general formula I as defined in claim 1.

26. Use of a compound of formula I as described in claim 1 for the manufacture of a medicament for the treatment of dysregulation of cytokines or for the modulation of cytokine disorders.

27. Use as claimed in claim 26 wherein the use is for manufacture of a medicament for treating neurodegenerative diseases.

28. Use as claimed in claim 27 wherein the medicament is for the arresting and reversing of neurodegeneration in multiple sclerosis of all types but particularly relapsing remitting, primary progressive and chronic progressive and the restoration, in part or completely, of neuronal integrity function such as measured, eg. By MRI or CAT scan or by EDSS score.

29. A method as claimed in claim 1 wherein the neurodegenerative disease involves demyelination.

30. A method as claimed in claim 1 wherein the treatment specifically arrests underlying neurodegeneration and restores neuronal function.

31. A method as claimed in claim 1 which normalises neuronal membrane composition with respect to  $\gamma$ -linolenic acid, dihomolinenic acid and arachidonic acid lipid content.

**32.** A method as claimed in claim 1 which restores healthy TGF- $\beta$ 1/TNF $\alpha$  ratios as measured from spontaneous release from peripheral blood mononuclear cell release.

**33.** A method as claimed in claim 1 wherein the disease is relapsing remitting multiple sclerosis, primary progressive multiple sclerosis or chronic progressive multiple sclerosis.

**34.** A method as claimed in claim 1 wherein the treatment is of cerebral impairment after stroke, head trauma and intracranial bleeding, Alzheimer's disease or Parkinson's disease where there is demyelination or neuronal damage.

**35.** A method as claimed in claim 1 wherein the lipid is administered for a duration and at a dose sufficient to maintain or elevate TGF- $\beta$ 1 levels in the patient to therapeutic levels.

**36.** A method as claimed in claim 1 wherein the lipid is administered for a duration and at a dose sufficient to maintain or elevate TGF- $\beta$ 1 levels in the patient to a TGF- $\beta$ 1/

TNF- $\alpha$  ratio released spontaneously from peripheral blood mononuclear cells isolated from the blood of a patient, after 18 months of daily dosing, of 0.4 to 3.0, at least 0.5, more preferably at least 0.75 and most preferably at least 1.

**37.** A method as claimed in claim 1 wherein the dose is such as to produce a TGF- $\beta$ 1/IL-1 $\beta$  ratio in PBMCs isolated from blood of a patient, after 18 months of daily dosing, of at least of at least 0.75.

**38.** A method as claimed in claim 1 wherein the amount of compound administered is between 0.5 and 30 grams, typically 3 to 5 grams, per day.

**39.** A method, use or composition as claimed in claim 1 wherein R<sup>3</sup> is  $\gamma$ -linolenyl or dihomog- $\gamma$ -linolenyl and the dose is between 1 and 10 grams/day.

**40.** A method, use or composition as claimed in claim 39 wherein the dose is between 2 and 10 grams/day.

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