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Selective in vivo anti-inflammatory action of the galactolipid monogalactosyldiacylglycerol

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Abstract

The thermophilic blue-green alga ETS-05 colonises the therapeutic thermal muds of Abano and Montegrotto, Italy. Following the isolation, purification and identification of monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG), sulphoquinovosyldiacylglycerol (SQDG) and phosphatidylglycerol from ETS-05, we here examine their in vivo anti-inflammatory activities. MGDG, DGDG and SQDG inhibit croton-oil-induced ear oedema in the mouse in a dose-dependent manner. Inhibition by MGDG is greater than that of the reference drug, betamethasone 17,21-dipropionate, and is largely abrogated following acyl group saturation. SQDG is the least potent of these glycoglycerolipids, and shows an early transient effect. In the in vivo carrageenan-induced paw oedema model in the mouse, the inhibitory effects are again dose dependent, with an enhanced efficacy of MGDG over DGDG, SQDG and the reference drug, indomethacin. These compounds are all less toxic than indomethacin. The selective and enhanced inhibitory effects of MGDG over DGDG indicate the mechanisms behind these in vivo anti-inflammatory actions.

Keywords: Monogalactosyldiglyceride; Inflammation; Croton oil; Carrageenan; Cyclooxygenase; Lipoxygenase

1. Introduction

Monogalactosyldiacylglycerols (MGDGs; Fig. 1A) and digalactosyldiacylglycerols (DGDGs; Fig. 1B) were originally isolated from plants over 40 years ago (see Kates, 1970). In contrast to the abundance of the phospholipids in membranes of animals and yeast, MGDGs and DGDGs represent some 75% of the total membrane lipids in plant leaves. These galactosyldiacylglycerols are thus especially important in the photosynthetic membranes of higher plants, and in algae and bacteria (see Dörmann and Benning, 2002). They have also been found at lower levels in animals, where they are enriched particularly in myelin and oligodendrocytes (Inoue et al., 1971; Pieringer et al., 1973, 1977; Morell and Toews, 1984). Similarly, the sulphoquinovosyldiacylglycerols (SQDGs; Fig. 1C), which are anionic glycoglycerolipids, are present in all photosynthetic plants, algae, cyanobacteria, and purple sulphur and non-sulphur bacteria (Barber and Gounaris, 1986). SQDGs are generally the most saturated of these glycoglycerolipids (Janero and Barrnett, 1981), although MGDG of the photosystem II reaction complex of spinach thylakoids is also highly saturated in comparison with that of the thylakoid membranes (Murata et al., 1990), indicating a functional relevance for the level of saturation of MGDG.

More recently, both natural and synthetic forms of these glycoglycerolipids have been shown to have specific biological activities, including anti-algal (Murakami et al., 1991), antiviral (Gustafson et al., 1989; Gordon and Danishefsky, 1992; Reshef et al., 1997; Loya et al., 1998), anti-tumour (Shirahashi et al.,

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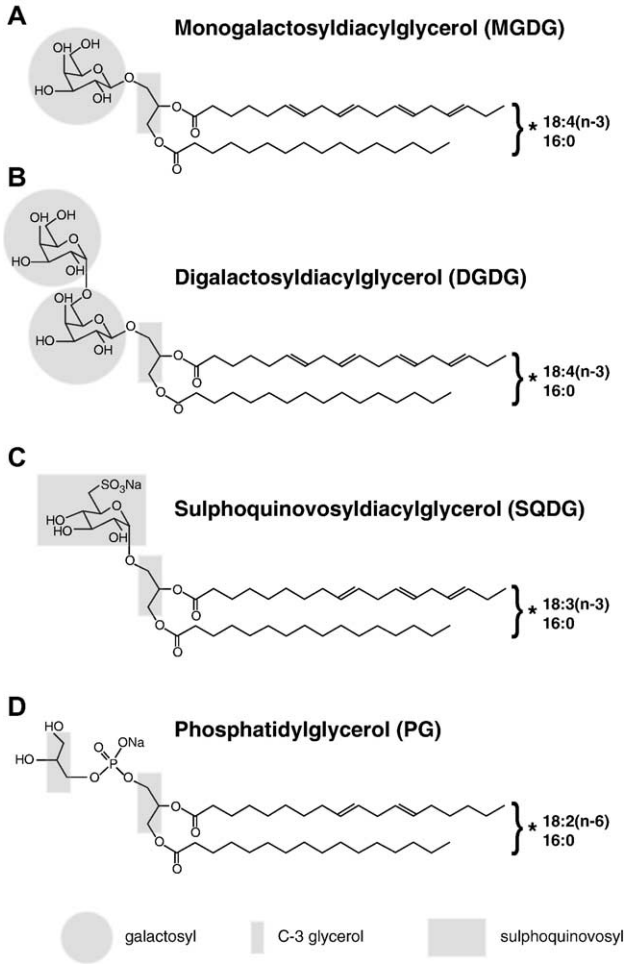

Fig. 1. Main structural components of the glycoglycerolipids used in this study (as isolated from the ETS-05 thermophilic blue-green alga). (A) Monogalactosyldiacylglycerol (MGDG); (B) digalactosyldiacylglycerol (DGDG); (C) sulphoquinovosyldiacylglycerol (SQDG); and (D) phosphatidylglycerol. The galactosyl, sulphoquinovosyl and glycerol moieties are as indicated. *The fatty acids indicated (which are here arbitrarily assigned to the *sn-1* and *sn-2* positions on the glycerol backbone) represent the two most abundant in each of the purified compounds from ETS-05 (Marcolongo et al., in press): 18:4(n-3), stearidonic acid; 18:3(n-3), linolenic acid; 18:2(n-6), linoleic acid; and 16:0, palmitic acid.

1993, 1996; Morimoto et al., 1995; Murakami et al., 1995; Tokuda et al., 1996; Sahara et al., 1997, 2002; Murakami et al., 2003), anti-inflammatory (Manez et al., 1999; Bergé et al., 2002; Larsen et al., 2003) and immunosuppressive activities (Matsumoto et al., 2000, 2002), and inhibition (Gad et al., 2001; Jayaprakasam et al., 2004) and promotion (Schmidt-Schultz and Althaus, 1994; Matsufugi et al., 2000b) of cell growth, and protection against cell death (Matsufugi et al., 2000a; Murakami et al., 2003). Furthermore, their HIV-1 antiviral actions are potentially due to inhibition of the viral reverse transcriptase (Reshef et al., 1997; Ohta et al., 1998), and a series of investigations of an SQDG (KM043; Ohta et al., 1998) also led to its definition as a mammalian DNA polymerase inhibitor (Ohta et al., 1998, 1999, 2000; Hanashima et al., 2000; Murakami et al., 2002).

The glycoglycerolipids of the thermophilic blue-green alga ETS-05, one of the most widespread and typical species of cyanobacteria of the therapeutic thermal muds of the Abano and Montegrotto hot springs in Padua, Italy, have recently been isolated, purified and structurally defined (Marcolongo et al., in press). Due to the increasing evidence that they can have anti-inflammatory activities (Manez et al., 1999; Bergé et al., 2002; Larsen et al., 2003), we have here determined the relative anti-inflammatory potencies of the purified MGDG, DGDG and SQDG from ETS-05 in two specific in vivo mouse models: croton-oil-induced ear oedema and carrageenan-induced paw oedema. We find that not only can MGDG in particular, and DGDG to a lesser extent, show enhanced anti-inflammatory activities and reduced toxicities over the positive controls, but also that the activity of MGDG is largely abrogated by saturation of its fatty acid chains. These data thus provide insights into the mechanisms behind the selective anti-inflammatory actions of these compounds.

2. Materials and methods

2.1. Animals

Male Balb/c mice (8–10 weeks old; Charles River, Milan, Italy) where housed under controlled temperature conditions (22±1 °C) with a fixed, 12-h, light–dark cycle and with food and water ad libitum. The procedures for their care conformed to the institutional guidelines that are in compliance with national (D.L. n. 116, G.U., suppl. 40, 18 February 1992) and international law and policies (European Economic Community Council Directive 86/609, OJ L 358, 1, 12 December, 1987; National Institutes for Health, USA, Guide for the Care and Use of Laboratory Animals; National Institutes for Health, USA, Publication No. 85-23, 1985; and Guidelines for Use of Animals in Biomedical Research).

2.2. Materials

MGDG, DGDG, SQDG and phosphatidylglycerol were chloroform/methanol/water (100:50:7, v/v) extracted from cultures of the ETS-05 cyanobacterium from the thermal muds of Abano and Montegrotto, Padua, Italy (Marcolongo et al., in press). Following their purification through n-hexane extraction and flash chromatography on silica gel, their structures were identified through their [^{1}H], [^{31}P] and [^{13}C] nuclear magnetic resonance spectra (Marcolongo et al., in press). Croton oil, type-IV lambda carrageenan, betamethasone 17,21-dipropionate and indomethacin were purchased from Sigma (Milan, Italy). All other salts and reagents were of the highest purities available, and were purchased from Sigma, unless otherwise specified.

2.3. Reduced MGDG

The reduced form of MGDG was obtained by catalytic hydrogenation of purified MGDG. Briefly, 100 mg MGDG was dissolved in 50 ml isopropanol, and 100 mg palladium catalyst on carbon was added. This reaction mixture was stirred at room temperature for 15 h under H_2 (with bubbling of H_2 gas). The

catalyst was then removed by filtration, and the filter washed with 10 ml of chloroform/methanol (2:1, v/v). The filtrate and washing solutions were combined and dried, and the product obtained was purified by flash chromatography on silica gel, with a mixture of chloroform/methanol/water/acetic acid (90:10:2.5:0.5; v/v) as eluent. The fractions containing the purified product were pooled, and after evaporation of the solvent, this gave a recovery of 90 mg of product (reduced MGDG) that had a solubility of 5 mg/ml in ethanol at 37 °C.

2.4. Croton oil ear oedema

For the croton oil ear oedema, MGDG and DGDG were dissolved in absolute ethanol (Carlo Erba, Milan, Italy), and SQDG and phosphatidylglycerol were dissolved in chloroform/ethanol 1:1 (v/v). The sample groups consisted of 4–5 animals, and the procedure was based on that described by Gabor (2003). Briefly, both ears of each mouse were treated on the back side with 3 μl of a solution of each compound in their volatile vehicle (or vehicle alone for controls), at the required concentrations. For each sample group, this procedure was repeated twice a day for 5 days, with the control group treated in parallel. As a positive control for comparative purposes, each sample group included animals treated with 3 μl 0.05% betamethasone 17,21-dipropionate, dissolved in the same vehicle as the tested compounds.

Thirty minutes after the last treatment application, 5 μl of a 20% croton oil solution in acetone was applied to each ear to induce inflammation. Ear thickness was measured immediately before this croton oil challenge ($t=0$) and at 24, 48 and 72 h after the croton oil application. The ear thickness measurements were taken using a digital micrometer (Biological Instruments, Varese, Italy), and the ear swelling was expressed as the differences in ear thickness (mm) between the baseline value ($t=0$) and those recorded at each time point. The data from ears that underwent any physical damage during the period of experimentation were excluded from the whole data set, and the final data from two or three independent experiments were combined to provide means ± S.E.M.

2.5. Carrageenan paw oedema

For carrageenan paw oedema, the alcoholic solutions of MGDG and DGDG were diluted in sterile, pyrogen-free 0.9% saline solution (Diaco Biomedicals SpA, Trieste, Italy) containing 0.5% Tween 80 (Merck, Schuchardt, Germany). SQDG was dissolved by sonication in dimethylsulphoxide (Carlo Erba, Milan, Italy), and then diluted in 0.9% saline. Essentially following previously described procedures (Henriques et al., 1987; Morris, 2003), sample groups of at least 6 mice received 50 μl subplantar injections of a 1% (w/v) carrageenan solution into the right hind paw. As carrageenan-induced oedema is known to have at least two phases (see Henriques et al., 1987; Morris, 2003; Posadas et al., 2004), the oedema was evaluated immediately before carrageenan injection ($t=0$) and 1, 2, 3, 4, 5, 6, 9, 24 and 48 h later. In the tabulation of the collected data, these time points have been grouped as means ± S.E.M. of (1 + 2) h, (3 + 4 + 5) h, (6 + 9) h and (24 + 48) h to reveal the phases of carrageenan-induced oedema. The paw volumes were measured using a water plethysmometer that was specifically modified for the measurement of small volumes (Ugo Basile, Milan, Italy). The non-steroidal anti-inflammatory drug (NSAID) indomethacin (10 mg/kg) was used as the positive-control reference drug, and the control groups were treated with their respective vehicles. Thirty min before carrageenan induction, the animals were treated s.c. with vehicle (control), indomethacin, MGDG, DGDG or SQDG, at the required concentrations. The resultant paw oedema was expressed as the differences between the volumes at each time point and those recorded at the baseline ($t=0$). The data from two independent experiments were combined to provide means ± S.E.M.

2.6. Data analysis

Statistical analyses were performed by analysis of variance for multiple comparisons, followed by *t*-tests.

3. Results

3.1. Croton oil ear oedema

For the croton-oil-induced ear oedema, a twice-a-day pre-treatment for 5 days was used to investigate the potential inhibitory effects of MGDG, DGDG, SQDG, phosphatidylglycerol (Fig. 1) and vehicles alone. Although there were no indications at any times prior to the application of croton oil of any effects of these compounds or the vehicles themselves (e.g. no visible reddening or swelling of or around the application site), this pre-treatment resulted in a significant small increase in ear thickness over the ethanol control (0.246 ± 0.002 mm) with 2% MGDG (0.277 ± 0.004 mm, $P<0.001$). Similarly, with the chloroform/ethanol control of 0.218 ± 0.002 mm, smaller significant pre-treatment increases in ear thickness were also seen with 2% SQDG (0.228 ± 0.002 mm, $P<0.01$) and 2% phosphatidylglycerol (0.233 ± 0.002 mm, $P<0.001$). These data represented increases of 12.9%, 4.0% and 6.0%, respectively, of the maximum croton-oil-induced response (obtained 24 h after its application; see Table 1). For MGDG, this small response did not vary significantly across the MGDG concentrations tested, and as it was also not seen for the positive control of 0.05% betamethasone (0.247 ± 0.004 mm in ethanol, $P=0.732$; 0.216 ± 0.003 mm in chloroform/ethanol, $P=0.473$) and at 2% DGDG (0.242 ± 0.004, $P=0.352$), it appeared not to be vehicle-related.

Table 1 shows the full range of the dose-related effects of these compounds on the ear inflammatory response 24, 48 and 72 h from croton oil application. As can be seen from the parallel controls under each treatment group, the croton-oil-induced oedema peaked from 24 to 48 h, before resolving some 96 h after croton oil application (data not shown). In the case of the positive control of 0.05% betamethasone, the combination of the full data sets in Table 1 demonstrates 25.0%

Table 1
Concentration dependence of inhibition of the croton-oil-induced ear inflammation by the glycoglycerolipids

	Time [a] (h)	Control [b]	Betamethasone [c] (0.05%)	0.5% [c]	1.0% [c]	2.0% [c]
MGDG	24	0.246±0.008 [d] (16)	0.195±0.011 [e] (22)	0.281±0.008 (12)	0.231±0.011 (12)	0.220±0.008 (18)
	48	0.238±0.006	0.201±0.007 [e]	0.237±0.009	0.184±0.014 [e]	0.151±0.012 [e, f]
	72	0.216±0.010	ND	0.142±0.043 [e]	0.097±0.012 [e]	0.101±0.010 [e]
DGDG	24	0.237±0.008 (28)	0.191±0.009 [e] (18)	0.245±0.011 (20)	0.231±0.012 (18)	0.190±0.009 [e] (20)
	48	0.253±0.006	0.181±0.007 [e]	0.220±0.009 [g]	0.199±0.014 [e]	0.170±0.006 [e]
	72	0.244±0.010	ND	0.171±0.006 [e]	0.160±0.011 [e]	0.140±0.008 [e]
SQDG	24	0.244±0.007 (12)	0.170±0.011 [e] (10)	0.222±0.005 (13)	0.209±0.004 [e] (13)	0.197±0.005 [e] (13)
	48	0.213±0.013	0.166±0.012	0.224±0.006	0.182±0.008	0.162±0.007 [g]
	72	0.177±0.015	ND	0.185±0.020	0.135±0.006	0.115±0.003 [e]
PG	24	0.238±0.008 (13)	0.168±0.006 [e] (10)	0.230±0.004 (13)	0.216±0.003 [g] (14)	0.202±0.005 [e] (14)
	48	0.225±0.008	0.197±0.010	0.235±0.006	0.199±0.014	0.181±0.012 [g]
	72	0.170±0.008	ND	0.184±0.009	0.157±0.010	0.144±0.010

The data shown are means±S.E.M. from two or three independent experiments; the *n* numbers are given with the 24 h data (in parentheses) for each individual group.
[a] Time from croton oil application.
[b] Vehicles alone: ethanol for MGDG and DGDG, ethanol/chloroform (1:1, v/v) for SQDG and phosphatidylglycerol (PG).
[c] In respective vehicle: ethanol for MGDG and DGDG, ethanol/chloroform (1:1, v/v) for SQDG and phosphatidylglycerol (PG).
[d] All data given as increases in ear thickness over $t=0$ for each group (see text). ND=not determined, due to necrosis of preparation; see text.
[e] Significantly different ($P<0.001$) from respective controls.
[f] Significantly different ($P<0.001$) from respective betamethasone-positive control.
[g] Significantly different ($P<0.01$) from respective controls.

(±2.9%) and 19.7% (±3.6%) inhibition of the croton-oil-induced inflammation after 24 h and 48 h, respectively. Of note, at 72 h, the betamethasone effect was such that the associated necrosis did not allow for further accurate ear-thickness measurements.

All four of the test compounds inhibited this croton-oil-induced oedema in a concentration-dependent manner (0.5%–2.0%), although higher concentrations of MGDG that were initially tested (up to 5%; data not shown) were not able to significantly increase the anti-inflammatory effect of 2% MGDG. A direct comparison of the 2% data in Table 1 demonstrates that although MGDG showed the smallest effect after 24 h, beyond 48 h it proved to be the most potent of these compounds, showing some 53% inhibition of the croton-oil-induced oedema by 72 h. While 2% DGDG and SQDG also showed increasing abilities to inhibit this inflammatory response (43% and 35% after 72 h, respectively), 2% phosphatidylglycerol showed a much reduced effect that lost its significance at this time. Of particular note, 2% MGDG was also the only one of these compounds to have a significantly greater inhibitory effect than 0.05% betamethasone ($P<0.001$), which was specifically seen 48 h from croton oil administration.

Following the isolation and purification of these four compounds from ETS-05, an analysis of their fatty acid content revealed the major constituent to be palmitic acid (16:0) (Marcolongo et al., in press). However, with MGDG and DGDG, as opposed to SQDG and phosphatidylglycerol, there was also a marked presence of polyunsaturated fatty acids (35–40% vs. ~10% in SQDG; Marcolongo et al., in press), and of 18:4(n-3) (stearidonic acid) in particular (MGDG=30%, DGDG=25%, and SQDG=1.5%; Marcolongo et al., in press). This high C18:4 content in MGDG and DGDG as compared with SQDG is also in agreement with a further recent fatty acid analysis of these glycoglycerolipids from the green alga *Ulva fenestrata* (MGDG=24%, DGDG=24%, and SQDG=1.6%; Sanina et al., 2004). Thus we also investigated the effect of the saturation (by catalytic reduction) of the fatty acids of MGDG on its anti-inflammatory activity in this croton-oil-induced ear oedema model. As seen in Table 2, in this series of experiments, 1% MGDG caused a significant reduction ($P<0.01$) in the anti-inflammatory effects of the control group after 24 h and 48 h. In this direct comparison, the anti-inflammatory effect of the reduced form of MGDG (Table 2), as compared to MGDG, was largely abrogated (with 6.5% vs. 19% maxima, respectively), although this was only significantly different at the earliest time point (24 h; $P<0.01$). These data thus indicate that the degree of saturation of the fatty acid chains of MGDG also has an important role in the anti-inflammatory activity of this compound.

Table 2
Comparison of inhibition of the croton-oil-induced ear inflammation by MGDG and reduced MGDG

	Time [a] (h)	Mean ear swelling (mm±S.E.M.)	Reduction in control (%)
Control [b]	24	0.220±0.005 (18)	–
	48	0.227±0.009	–
	72	0.172±0.010	–
MGDG (1%)	24	0.178±0.004 [c] (20)	19.1 [c]
	48	0.184±0.008 [c]	18.9 [c]
	72	0.158±0.012	8.1
Reduced MGDG (1%)	24	0.207±0.009 [d] (20)	5.9 [d]
	48	0.212±0.012	6.6
	72	0.182±0.018	–

The data shown are means±S.E.M. from three independent experiments; the *n* numbers are given with the 24 h data (in parentheses) for each individual group.
[a] Time from croton oil application.
[b] Vehicle alone (ethanol).
[c] Significantly different ($P<0.01$) from control.
[d] Significantly different ($P<0.01$) from MGDG control.

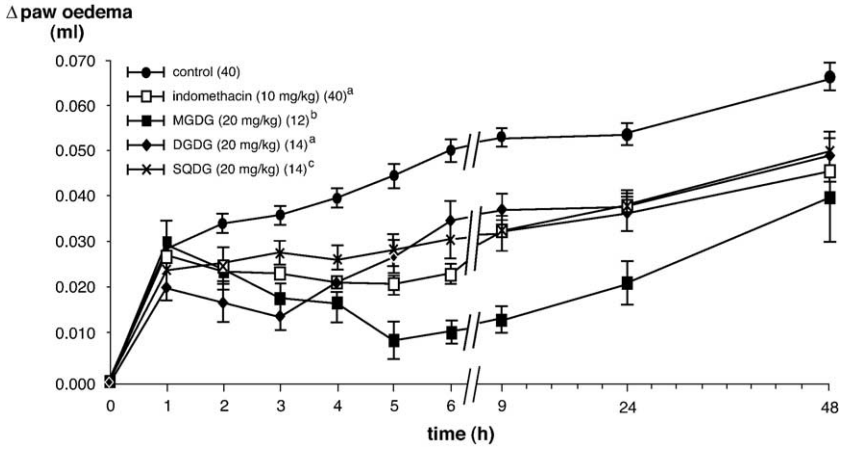

Fig. 2. Inhibition of carregeenan-induced paw oedema by the glycoglycerolipids. Increases in paw volume measurements after injection of 1% carrageenan 30 min after pretreatment with vehicle (control), 10 mg/kg indomethacin or 20 mg/kg solutions of the glycoglycerolipids (as indicated). The data shown are means ± S.E.M. from two independent experiments; the *n* numbers are given in brackets in the key. The greater control and indomethacin *n* numbers arise as these data have been combined across all of the experiments. [a]Indomethacin, DGDG: significantly different ($P<0.002$) vs. control from 2 to 48 h inclusive. [b]MGDG: significantly different ($P<0.005$) vs. control from 3 to 48 h inclusive, and vs. indomethacin from 5 to 24 h inclusive. [c]SQDG: significantly different ($P<0.003$) vs. control from 4 to 48 h inclusive.

3.2. Carrageenan paw oedema

The injection of carrageenan into the hind paw produced an inflammatory response that was quantified by measuring the changes in footpad volume by water displacement 1, 2, 3, 4, 5, 6, 9, 24 and 48 h afterwards. As can be seen from the full control data in Fig. 2, and as has been analysed in some detail more recently (Posadas et al., 2004), the inflammatory response induced by 1% carrageenan showed an initial stage that peaked within the first 2–3 h, and a later amplification phase from 4 to 24 h. The response then increased slowly up to the final 48-h time point shown, before decreasing towards the original untreated control (data not shown).

From the pooled time-point data given in Table 3, it can be seen that the prior s.c. treatment with MGDG showed significant dose-dependent anti-inflammatory effects on carrageenan-induced paw oedema in the concentration range tested (10 mg/kg to 40 mg/kg). Similar, but reduced, significant dose-dependent effects were seen for DGDG and SQDG (5–20 mg/kg and 10–40 mg/kg, respectively; Table 3). In a full time-point expansion of the pooled 20 mg/kg data used for Table 3 (at which concentration a maximal MGDG response was obtained), both MGDG and DGDG showed a reduction in the foot-pad oedema with respect to the control that was significant from 3 h ($P<0.005$) and 2 h ($P<0.002$) after carrageenan injection, respectively (Fig. 2). SQDG had smaller, but essentially similar, effects at these times. These early effects of the glycoglycerolipids thus initially paralleled those of the positive control of 10 mg/kg s.c. indomethacin; furthermore, they all persisted for the full 48 h duration of the experiments.

Table 3
Concentration dependence of inhibition of the carrageenan-induced footpad inflammation by the glycoglycerolipids

	Time[a] (h)	Control[b]	Indomethacin[c] (10 mg/kg)	5 mg/kg[c]	10 mg/kg[c]	20 mg/kg[c]	40 mg/kg[c]
MGDG	(1+2)	0.035±0.002[d] (24)	0.034±0.003 (24)	nd	0.033±0.003 (12)	0.027±0.003 (22)	0.033±0.003 (12)
	(3+4+5)	0.044±0.002 (36)	0.021±0.002[e] (36)	nd	0.026±0.002[e] (18)	0.015±0.002[e] (33)	0.022±0.003[e] (18)
	(6+9)	0.048±0.002 (24)	0.024±0.003[e] (24)	nd	0.037±0.004 (12)	0.012±0.002[e, f] (22)	0.020±0.004[e] (12)
	(24+48)	0.056±0.004 (24)	0.046±0.004 (24)	nd	0.048±0.004 (12)	0.028±0.005[e, f] (22)	0.030±0.005 (12)
DGDG	(1+2)	0.030±0.004 (28)	0.022±0.002 (28)	0.032±0.003 (28)	0.029±0.003 (28)	0.024±0.003 (28)	nd
	(3+4+5)	0.041±0.003 (42)	0.022±0.003[e] (42)	0.035±0.003 (42)	0.028±0.003 (42)	0.024±0.002[e] (42)	nd
	(6+9)	0.058±0.002 (28)	0.032±0.002[e] (28)	0.044±0.004 (28)	0.044±0.004 (28)	0.038±0.003[e] (28)	nd
	(24+48)	0.062±0.002 (28)	0.036±0.002[e] (28)	0.044±0.003 (28)	0.049±0.004 (28)	0.043±0.004 (28)	nd
SQDG	(1+2)	0.026±0.002 (28)	0.023±0.002 (28)	nd	0.023±0.003 (28)	0.025±0.002 (28)	0.027±0.002 (26)
	(3+4+5)	0.035±0.002 (42)	0.023±0.002[e] (42)	nd	0.024±0.002[e] (42)	0.028±0.002 (42)	0.017±0.002[e] (39)
	(6+9)	0.047±0.003 (28)	0.028±0.002[e] (28)	nd	0.034±0.003[e] (28)	0.031±0.003[e] (28)	0.021±0.002[e] (26)
	(24+48)	0.060±0.003 (28)	0.045±0.002[e] (28)	nd	0.047±0.003 (28)	0.045±0.003[e] (28)	0.046±0.003 (26)

The data shown are means ± S.E.M. from two independent experiments, with the time points combined as indicated; the resultant *n* numbers are given (in parentheses) for each individual group.

[a] Combined times from carrageenan injection (see Methods).
[b] Vehicle alone (see Methods).
[c] In respective vehicle (see Methods).
[d] All data given as increases in volume over $t=0$ (0.112±0.001 ml). nd = concentration not tested.
[e] Significantly different ($P<0.001$) from respective controls.
[f] Significantly different ($P<0.01$) from respective indomethacin-positive control.

Of particular interest, however, is that MGDG showed a further inhibitory action over that of the 10 mg/kg indomethacin positive control, a difference that reached significance 5 h after the carrageenan injection ($P<0.05$ vs. indomethacin; Fig. 2). This enhanced inhibition of the second phase of the carrageenan oedema response was not seen with DGDG and SQDG, and it persisted up to 24 h, although it did not significantly affect the maximal oedema seen in the presence of any of the tested compounds after 48 h (Fig. 2). Of note, the highest dose of indomethacin originally tested (18 mg/kg; data not shown) was reduced to 10 mg/kg here due to experimental toxicity; at 18 mg/kg, indomethacin showed lethality within 24 h of application, rising to a loss of 50% of the treated animals after 72 h. In contrast, the glycoglycerolipids were essentially non-toxic, with MGDG remaining non-lethal and showing no local effects on injection even up to 80 mg/kg, the highest concentration tested (data not shown).

The evaluation of these three glycoglycerolipids in this second in vivo inflammation model thus confirmed the reduced efficacy of SQDG in comparison with MGDG and DGDG that was seen in the ear inflammation model. Indeed, this foot-pad inflammation model also demonstrated a significantly enhanced efficacy of MGDG over DGDG that was apparent, but not significant, in the ear inflammation model (Table 1). As this difference between MGDG and DGDG was clearly seen from 5 h after the carrageenan injection, these two similar glycoglycerolipids (Fig. 1) are both able to inhibit the first phase of carrageenan-induced inflammation to an equal extent (by ~50%; Fig. 2), but only MGDG is able to inhibit the onset of the later inflammatory response (reaching ~80% maximal inhibition). At the same time, the ability for MGDG to show such a sustained and large inhibition of this carrageenan-induced inflammation shows this to be a strong and specific affect, rather than one that might arise as a reflection of the low level of ear thickening that was caused by MGDG itself in the croton oil model (as a potential effect from a secondary site of inflammation).

4. Discussion

Here we have demonstrated that the purified glycoglycerolipids from the cyanobacterium ETS-05 (Marcolongo et al., in press) show anti-inflammatory activities in two in vivo models of inflammation: croton-oil-induced mouse ear oedema and carrageenan-induced mouse footpad oedema, which have revealed a selective efficacy and specificity of MGDG. In previous studies that have described anti-inflammatory activities of members of this family of glycoglycerolipids, their sources and definitions have to date been particularly variable. These have included the anti-inflammatory effects of an eicosapentaenoic-acid (20:5n-3)-containing MGDG (from the marine microalga *Chlorella minutissima* (Winget, 1997), a DGDG from the Jordanian medicinal plant *Inula viscosa* (Manez et al., 1999), an SQDG fraction from the red microalga *Porphyridium cruentum* (Bergé et al., 2002), and an MGDG from the rose hip (*Rosa canina*; Larsen et al., 2003). Similarly, the inflammation assay systems have been varied, and included croton-oil-induced oedema, 12-*O*-tetradecanoylphorbol-13-acetate-induced oedema, phorbol-myristate-acetate-induced superoxide anion generation in peritoneal leukocytes, and chemotaxis of human peripheral neutrophils in vitro, respectively. Other reports of other activities of these compounds have been similarly variable in origins and in the activities defined.

The previous use of a defined MGDG structure and its effects as an anti-inflammatory agent have been limited to an enriched eicosapentaenoic-acid-containing MGDG preparation (~60% eicosapentaenoic acid) from the marine microalga *Chlorella minutissima* (Winget, 1997). In the present study, we aimed to combine the fuller definition of the structures with a wider definition of their anti-inflammatory activities by using the MGDG, DGDG and SQDG that were recently isolated from the thermophilic blue-green alga ETS-05 and that have been defined in terms of their fatty acid contents (Marcolongo et al., in press). Thus we have tested these glycoglycerolipids in two in vivo mouse models of inflammation.

Our data initially show that MGDG, DGDG, SQDG and phosphatidylglycerol extracted and purified from cultures of the ETS-05 alga have different anti-inflammatory activities. In particular, MGDG was the most active of these compounds in both of the in vivo inflammation models used here, while phosphatidylglycerol was the least. This is in agreement with the previous study on an eicosapentaenoic-acid-containing MGDG, which demonstrated a greater anti-inflammatory activity of an eicosapentaenoic-acid-containing MGDG over phosphatidylglycerol (Winget, 1997). Furthermore, this previous study also showed that structural changes to the fatty acid content of MGDG influenced its anti-inflammatory activity, in that the substitution of eicosapentaenoic acid for different carbon chain lengths or levels of saturation, such as linolenic-acid (18:3)-containing MGDG, reduced the efficacy of this compound (Winget, 1997). In agreement with this, we have also shown that the catalytic reduction (and hence saturation) of MGDG largely abrogated its anti-inflammatory activity in the croton oil oedema assay.

As indicated above, MGDG was the most potent of our compounds in both inflammation models. However, of added interest in the carrageenan paw oedema assay, MGDG also showed a potency beyond that of the 10 mg/kg indomethacin positive control. Indeed, although MGDG was used at a higher concentration (20 mg/kg), we also observed in our preliminary trials that with an equivalent concentration of indomethacin (18 mg/kg), there was a toxicity problem, where 50% of these indomethacin-treated mice died within 72 h of treatment. Furthermore, the surviving animals demonstrated clear signs of toxicity (ruffled fur, evident loss of weight, difficulties in movement; not shown). Thus, not only is MGDG more effective as an anti-inflammatory in this model, but it is also much less toxic than indomethacin. Similarly, during the positive control treatment in the croton-oil model, 2% MGDG not only had a significantly greater inhibitory effect than the positive control of 0.05% betamethasone after 48 h, but it also did not lead to the continued betamethasone-induced deterioration of the preparation that prevented the 72-h data measurements for

the positive control groups. In contrast, none of the animals treated with the cyanobacterium glycoglycerolipids showed signs of toxicity in either model, even at the highest doses used.

It has also been suggested that the anti-inflammatory activity of an eicosapentaenoic-acid-containing MGDG could result from the metabolic release of eicosapentaenoic acid, allowing this to enter the cellular phospholipid pools and function as a substrate in the metabolic pathways of arachidonic acid (20:4 (n-6); the eicosanoid pathway) (Winget, 1997). The classical consideration of the triggering of inflammation under these conditions would involve the group IVA, calcium-sensitive, 85-kDa, cytosolic phospholipase A_2 ($PLA_2\alpha$; for a review of PLA_2s see Balsinde et al., 2002), which would be activated to release arachidonic acid, and potentially also eicosapentaenoic acid here, from the cellular phospholipids. The cyclooxygenases (COX-1 and COX-2) and lipoxygenases (5-LOX, 12-LOX and 15-LOX) then catalyse the metabolism of arachidonic acid to the prostaglandins and thromboxanes, and to the leukotrienes, hydroxy fatty acids and lipoxins, respectively, all of which can have inflammatory activities. Eicosapentaenoic acid and, in general, fatty acids that are structurally related to arachidonic acid, can also be used as COX and LOX substrates, which could lead to a suppression of the biosynthesis of the arachidonic-acid-derived eicosanoids (see Larsson et al., 2004, for review). The mechanisms behind these effects remain to be fully elucidated, although as is further discussed below, recent evidence has suggested a varying role of the PLA_2s (Gilroy et al., 2004) and suppression of the COX-2 pathway (Larsson et al., 2004). In the present study, the anti-inflammatory effects of these glycoglycerolipids would appear unlikely to be due specifically to the release of their fatty acids for two specific reasons: (i) the low levels of the glycoglycerolipids needed for these inhibitory effects (maximal effects seen at 2%), in competition with the higher concentrations of arachidonic acid needed to produce inflammatory effects (20%; Gabor, 2003); and in particular, (ii) the differential effects of MGDG and DGDG in the carrageenan-induced oedema, despite their very similar fatty acid contents, which include ~80% of a combination of the 16:0 (palmitic acid) and 18:4(n-3) chains in this cyanobacterium MGDG and DGDG (Marcolongo et al., in press).

As has been known for many years, and as considered in further detail in more recent investigations in the mouse (Posadas et al., 2004) and the rat (Nantel et al., 1999), the carrageenan inflammation model shows a biphasic inflammation response that varies according to carrageenan concentration (Nantel et al., 1999; Posadas et al., 2004), and mouse age (3 to 8 weeks old; Posadas et al., 2004) and type (CD1 vs. C57BL/6J; Posadas et al., 2004). With the 8- to 10-week-old Balb/c mice and 1% carrageenan used in the present study, we clearly see two phases of response after the carrageenan injection: the first, acute phase that peaks within 2 to 3 h; and the second, later phase from 4 to 24 h. Indomethacin, the NSAID and non-specific COX inhibitor, inhibited the acute inflammation by ~35% after 3 h, while essentially blocking the second phase of the carrageenan response. With DGDG, a greater block of the acute phase was seen (~60% at 3 h), although it then displayed a parallel time course to the carrageenan control (see Fig. 2), indicating that it had no effects on the second phase of inflammation. In contrast, while MGDG showed an intermediate inhibition of the acute inflammation phase (~50%) between that seen with indomethacin (~35%), SQDG (~20%) and DGDG (~60%), its inhibition continued to increase with the onset of the second phase. Therefore, by 5 h after carrageenan injection, the MGDG inhibition (80%) was significantly greater than that of indomethacin (53%), DGDG (40%) and SQDG (36%), although this enhanced anti-inflammatory effect of MGDG was gradually lost over the following 40 h.

Consideration of the mechanisms behind these two phases of carrageenan-induced inflammation can provide some insights into this selective inhibition. During the acute phase, it has been shown that neutrophils are the predominant cells, while there is an accumulation of macrophages, eosinophils and lymphocytes during the second phase (Henriques et al., 1987). Many further studies have shown the production of a wide range of inflammatory agents, which include not just the products of the COX and LOX activities (see above), but also histamine, serotonin, bradykinin, free radicals and nitric oxide. However, the first stage of the enzymic activation during carrageenan-induced oedema that needs to be considered is that of the PLA_2 activation that leads to the production of arachidonic acid. Thus these glycoglycerolipids could have direct actions as inhibitors of this PLA_2 activity, rather than being competitive substrates. In support of this, Vishwanath et al. (1996) extracted and isolated several structurally related glycoglycerolipids from the field thalli of the brown marine macroalga *Fucus serratus* L. and assessed their effects on the activities of the group I and group II, low molecular mass, secretory PLA_2s. While their MGDG had no effects here, their DGDG inhibited and their SQDG activated these secretory PLA_2s (Vishwanath et al., 1996). They also indicated that these effects depended on the different structural elements: namely, on the glycerol–sugar backbone and on the type and degree of saturation of the fatty acid groups.

However, as indicated above, the classical thinking around inflammatory responses would not involve these secretory PLA_2s. Despite this, a more recent report investigated the full range of PLA_2s involved in this biphasic inflammation response to carrageenan using a range of specific subtype-selective PLA_2 inhibitors (Gilroy et al., 2004). In doing so, doubt must now be cast on the classical thinking, as they showed that the first, acute phase appeared to be driven by the group VI, calcium-insensitive PLA_2. This then switched during the second phase of the response, with the sequential expression of the secretory PLA_2s (groups IIA and V) and then cytosolic $PLA_2\alpha$, with the latter providing the arachidonic acid substrate for the later-expressed COX-2 (see below). A combination of this selective inhibition of secretory PLA_2s by DGDG (Vishwanath et al., 1996) and potential involvement of secretory PLA_2s in "activating" the second phase of carrageenan-induced oedema (Gilroy et al., 2004) would thus imply that DGDG should selectively inhibit the second inflammation phase. However, as can be seen in Fig. 2, in our hands it was MGDG that displayed this action, with DGDG clearly having no effects after the initial 2 to 3 h of carrageenan injection. Thus it would appear unlikely

that the actions of the glycoglycerolipids in the present study arise from such selective inhibition of the PLA_2s.

Following on from the initial production of arachidonic acid during the inflammatory response, the main enzymatic elements that have been considered in detail with respect to carrageenan effects are COX-1 and COX-2, and the nitric oxide synthases. Thus, a recent analysis of the two phases of the carrageenan-induced inflammatory response in mice monitored the expression of endothelial nitric oxide synthase (NOS), inducible NOS, COX-1 and COX-2 in parallel with nitric oxide (NO_x; as a measure of nitric oxide synthase activity; nitrite plus nitrate) and prostaglandin E_2 (as a measure of COX activity) production and tissue myeloperoxidase levels (as a measure of neutrophil infiltration) (Posadas et al., 2004). For the nitric oxide synthases, this study showed that the acute production of NO_x arises from constitutive endothelial NOS expression, while a later increase in endothelial NOS and induction of inducible NOS (after 6 h) is responsible for the second phase of NO_x production. Similarly, the first of the two phases of prostaglandin E_2 production arises from the constitutive COX-1 activity, and the second phase is due to both COX-1 and the induced COX-2 (after 24 h; potentially by the prostaglandins produced by COX-1; Siqueira-Junior et al., 2003). The myeloperoxidase levels peaked at 24 h (Posadas et al., 2004).

A comparison of these time-related effects on COX and nitric oxide synthase activities would thus indicate that our MGDG inhibition of the second phase of carrageenan-induced oedema could be due to a selective effect on the induced COX-2 activity and/or a selectivity towards endothelial NOS inhibition. Considering that COX-2 appears responsible for the elevated production of prostanoids that occurs at sites of both disease and inflammation, and that many of the side-effects of the less specific COX inhibitors appear to be due to their inhibition of the constitutive COX-1 activity, efforts continue to be made towards the development of new synthetic drugs that are targeted directly against COX-2 (see Warner and Mitchell, 2004, for review). Thus it will be of importance to investigate the selective effects of these glycoglycerolipids in specific in vitro enzyme activity assays in order to determine whether MGDG from these ETS-05 algae is indeed a specific COX-2 inhibitor. Similarly, with carrageenan also being able to induce the elevated expression of 5-LOX in the rat (Turesin et al., 2003), and with the 5-LOX knock-out mouse showing that 5-LOX modulates neutrophil infiltration in carrageenan-induced acute lung inflammation (Cuzzocrea et al., 2003), the effects of these glycoglycerolipids now need to be determined on this full range of activities.

Although a recent study has stressed the importance of NOS activity in the second phase of carrageenan-induced inflammation in the mouse through the use of nitric oxide donors (Fernandes and Assreuy, 2004), the above considerations point to a potential preferential COX-2 (over COX-1) inhibition for our MGDG. Indeed, another recent study has looked directly at the abilities of three MGDGs to inhibit COX-1 and COX-2 in vitro and the growth of a series of cancer cell lines in culture (Jayaprakasam et al., 2004). Their MGDGs were isolated from the leaves of *Amaranthus tricolor*, a plant used as a vegetable in Southeast Asia, and their main fatty acid constituent was 18:3(n-3) (linolenic acid): 100% in compound 1 and 50% in compounds 2 and 3 (along with 50% 16:0, palmitic acid, and 18:0, stearic acid, respectively) (Jayaprakasam et al., 2004). This thus contrasts heavily with the fatty acid content of the MGDG in the present study despite its high 16:0 content (52%); our MGDG contained no 18:0 and only ~3% 18:3(n-3), with 30% being 18:4(n-3) instead (Marcolongo et al., in press). However, of interest to our present study is that Jayaprakasam et al. (2004) indicated that all three of their MGDGs can inhibit both COX-1 and COX-2 activities in vitro. As they also note that the degree of saturation appears to have a role in both the COX-1/2 and tumour cell growth inhibition, although no direct comparisons are possible with the present data, their results do parallel our present investigations into the mechanisms behind these potential anti-cancer activities of our MGDG in particular.

In conclusion, a combination of the present study and previous investigations into these naturally occurring plant glycoglycerolipids has allowed the initial definition of the importance of both their galactosyl and acyl groups in their anti-inflammatory actions. With the more recent and precise definitions of the fatty acid contents of these glycoglycerolipids from different sources (Guella et al., 2003; Jayaprakasam et al., 2004; Sanina et al., 2004; Marcolongo et al., in press), and the widening of the potential biological uses of both the glycoglycerolipids and their associated fatty acids (Wen and Chen, 2003), the mechanisms behind their full structure-activity relationships across their complex range of structures and the complex range of the enzymic activities involved in the inflammation response need to be better defined. This will thus allow the promotion of their use as potential naturally occurring templates for future drug development.

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