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Xanthones as Inhibitors of Microsomal Lipid Peroxidation and TNF-α Induced ICAM-1 Expression on Human Umbilical Vein Endothelial Cells (HUVECs)

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Abstract—Xanthones bearing different functionalities, namely 1-hydroxyxanthone (**1**), 3-hydroxyxanthone (**2**), 1,4-dihydroxyxanthone (**3**), 2,6-dihydroxyxanthone (**4**), 1,2-diacetoxyxanthone (**5**), 2,6-diacetoxyxanthone (**6**), 3-methoxyxanthone (**7**), 1,3,7-trimethoxyxanthone (**8**) and 1,5-dihydroxy-6-methoxyxanthone (**9**) were synthesised and examined for their effect on nicotinamide adenine dinucleotide phosphate (NADPH)-catalysed liver microsomal lipid peroxidation and on tumour necrosis factor-α (TNF-α) induced expression of intercellular adhesion moledule-1 (ICAM-1) on endothelial cells, with a view to establish structure–activity relationship. Hydroxy- and acetoxyxanthones showed potent inhibitory effects on NADPH-catalysed lipid peroxidation and TNF-α induced expression of ICAM-1 on endothelial cells.

Introduction

Adhesion of leukocytes to the endothelium is a primary step during inflammation. During inflammation, the expression of cell adhesion molecules is upregulated on the surface of endothelial cells thus altering the adhesive property of the vasculature and allowing indiscriminate infiltration of the leukocytes across the blood vessels.[1] Inflammatory cytokines like tumour necrosis factor-α (TNF-α) and IL-1 upregulate the expression of intercellular adhesion molecule-1 (ICAM-1) on endothelial cells.[2] Accumulating evidence suggests that TNF-α increases the production of reactive oxygen intermediates such as superoxides, lipid peroxides and nitric oxide (NO), which act as second messengers during the TNF-α induced ICAM-1 expression.[3] The signals in the form of oxidised lipoproteins and reactive oxygen species lead to the activation of transcription factors such as nuclear factor-κB (NF-κB) that are involved in the regulation of expression of ICAM-1.[4] NF-κB is an oxidative stress responsive transcription factor, specially activated by peroxides.[5] Shear stress induced ICAM-1 expression on endothelial cells also involves activation of ROS acting as second messengers.[6] Also recent studies suggest that increased circulating lipid peroxides in pre-eclemptic women are responsible for increased ICAM-1 expression on endothelial cells.[7]

The compounds that inhibit lipid peroxidation and influence the generation of ROS in turn lead to the decreased expression of cell adhesion molecule and subsequently decreased inflammation hence found to be a useful therapeutic approach in various inflammatory diseases.[8] Treatment of endothelial cells with antioxidants is also shown to downregulate the expression of ICAM-1 on endothelial cells.[9] Studies by various groups using different models demonstrate that xanthones posses anti-inflammatory activity.[10,11] In the present study, for understanding the mechanisms underlying the anti-inflammatory activities of xanthones and for studying their structure–activity relationship, we have synthesized various hydroxy-, acetoxy- and methoxyxanthones. These xanthone derivatives have been analyzed for their ability to modulate the TNF-α

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induced expression of ICAM-1 and their effect on liver microsomal lipid peroxidation has also been studied. Of the several xanthones analysed, we found that hydroxyxanthones were more potent in inhibiting microsomal lipid peroxidation and TNF-α induced expression of ICAM-1 as compared to acetoxyxanthones, which in turn were more active than methoxylated and partially methoxylated xanthones.

Results

Xanthones inhibit the TNF-α induced expression of ICAM-1 on endothelial cells

The effect of nine different hydroxy-, acetoxy- and methoxyxanthones has been examined on the modulation of cytokine-induced expression of ICAM-1 in human endothelial cells (Fig. 1). The endothelial cells plated to confluence in 96-well plates were incubated with varying concentrations of xanthones (Table 1). The effect of these xanthones on the viability (determined by trypan blue exclusion test) and the morphology of the endothelial cells (observed under microscope) were tested. The maximal tolerable concentrations were found to be different for different xanthones. For further analysis, the concentrations at the maximal tolerable range were used. The effect of nine xanthones (Table 1) on TNF-α induced ICAM-1 expression was analyzed using cell-ELISA as detailed in the Experimental.

Table 1. Effect of xanthones on the TNF-α induced expression of ICAM-1 on endothelial cells

Serial No.	Compound name	Concentration (μg/mL)[a]	% Inhibition of ICAM-1 expression
1	1-Hydroxyxanthone	60	22.2
2	3-Hydroxyxanthone	66	13.7
3	1,4-Dihydroxyxanthone	65	86.0
4	2,6-Dihydroxyxanthone	18	40.9
5	1,2-Diacetoxyxanthone	75	42.4
6	2,6-Diacetoxyxanthone	100	23.8
7	3-Methoxyxanthone	46	0
8	1,3,7-Trimethoxyxanthone	13	0
9	1,5-Dihydroxy-6-methoxyxanthone	20	1.6

[a]The concentration levels of different compounds are based on their maximum tolerable concentrations by the cells.

Our results using cell-ELISA demonstrate that ICAM-1 was expressed at low levels on unstimulated endothelial cells and there was over 5-fold increase in its expression

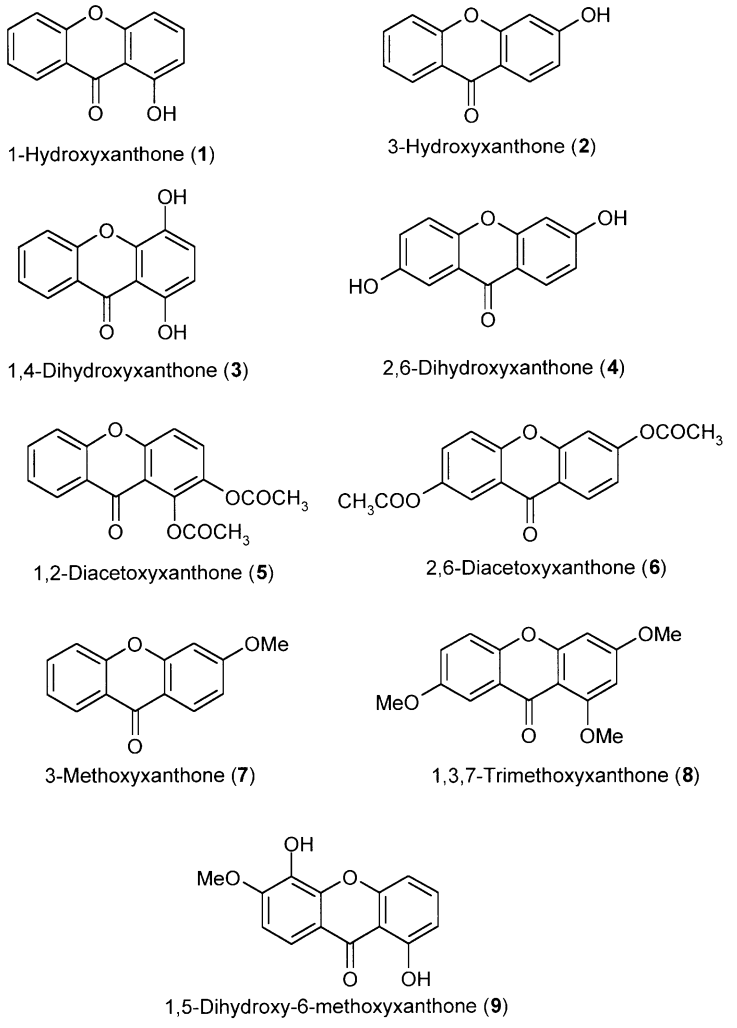

Figure 1. Structures of the xanthones tested.

upon stimulation with TNF-α (data not shown). Pretreatment of endothelial cells with xanthones had no effect on the constitutively expressed levels of ICAM-1, while they had varying effects on TNF-α induced ICAM-1 expression (Table 1). As the maximum tolerable concentrations used in these experiments are to some degree different, a direct comparison cannot be made. However, it has been found that 1,4-dihydroxyxanthone (**3**) inhibited the ICAM-1 expression by 86.0%. 2,6-Dihydroxyxanthone (**4**), 1,2-diacetoxyxanthone (**5**) and 2,6-diacetoxyxanthone (**6**) inhibited the ICAM-1 expression by 40.9, 42.4 and 23.8%, respectively (Table 1). Among the monohydroxylated xanthones, 1-hydroxyxanthone (**1**) inhibited the expression by 22.2% and 3-hydroxyxanthone (**2**) inhibited by 13.7%. The methoxylated xanthones, 3-methoxyxanthone (**7**), 1,3,7-trimethoxyxanthone (**8**) and partially methoxylated xanthone, 1,5-dihydroxy-6-methoxyxanthone (**9**) showed no effect upto the maximal tolerable concentrations tested.

Xanthones inhibit lipid peroxidation

Reactive oxygen species are primary signalling molecules in regulating the expression of ICAM-1 on endothelial cells and hence play an important role in various inflammatory diseases. As we observed that the xanthones inhibited ICAM-1 expression, we were interested in studying their effect on lipid peroxidation. The effect of these different hydroxy-, acetoxy- and methoxyxanthones was examined on the initiation of lipid peroxidation in liver microsomes. It has been observed from the results illustrated in Table 2 that the dihydroxylated xanthones **3** and **4** showed maximum inhibition of initiation of lipid peroxidation, followed by monohydroxylated xanthones **1** and **2**. The fully methoxylated xanthones **7** and **8** and hydroxy, methoxylated xanthone **9** did not show any appreciable inhibition of initiation of lipid peroxidation. Further, it has been found that the inhibition of initiation of lipid peroxidation by compound 5 was comparable to that of compound **1**.

Interestingly, we have observed that the inhibition patterns of nicotinamide adenine dinucleotide phosphate (NADPH) catalysed lipid peroxidation initiation and TNF-α induced ICAM-1 expression followed very similar trend (Fig. 2).

Discussion

We have in this investigation analyzed the xanthones for their ability to modulate TNF-α induced ICAM-1 expression and have also utilised the initiation step of NADPH-catalysed microsomal lipid peroxidation in order to examine their antioxidant property. As shown in Tables 1 and 2, hydroxy substitution on the xanthone nucleus is required for both antioxidant activity and ICAM-1 inhibitory activity. For example, the hydroxylated xanthones **1**–**4** showed high to moderate activity. The dihydroxyxanthones **3** and **4** show increased activity as compared to monohydroxyxanthones, that is **1** and **2**, indicating that increase in the number of hydroxy substituents on the xanthone nucleus enhances the antioxidant as well as ICAM-1 inhibitory activity. The activity of hydroxyxanthones may be because of two reasons: (a) facile oxidisable nature of such compounds resulting in the formation of quinones having stable quinonoid structures (Fig. 3), and (b) the ability to form stable phenoxy radicals. This proposition is further supported by the observation that 1,4-dihydroxyxanthone has better activity as compared to 2,6-dihydroxyxanthone as the oxidized quinone form (**X**) of the former compound is more stable than the quinone form (**XI**) of the latter (Fig. 3). Further, compound **5** exhibited comparable activity for inhibition of initiation of lipid peroxidation to that of **1** (Table 2); the two diacetoxyxanthones **5** and **6** exhibited comparable activity for the inhibition of ICAM-1 expression to that of **4** and **1**, respectively (Table 1). The lipid peroxidation and ICAM-1 inhibitory activity of diacetoxyxanthones **5** and **6** may be because of in-situ enzymatic deacetylation of these compounds leading to the formation of the corresponding dihydroxyxanthones **XII** and **4** (Fig. 3), respectively. The dihydroxyxanthones thus formed may be oxidized to the corresponding quinonoid xanthones, the species that may really be responsible for the observed activities (Fig. 3). The stability of the *ortho*-quinonoid

Table 2. Effect of xanthones on NADPH-dependent inhibition of lipid peroxidation initiation

Serial No.	Compound name	NADPH-dependent lipid peroxidation initiation, percent of the control[a]
1	1-Hydroxyxanthone	28
2	3-Hydroxyxanthone	38
3	1,4-Dihydroxyxanthone	60
4	2,6-Dihydroxyxanthone	43
5	1,2-Diacetoxyxanthone	30
6	2,6-Diacetoxyxanthone	–
7	3-Methoxyxanthone	2
8	1,3,7-Tri-methoxyxanthone	0
9	1,5-Dihydroxy-6-methoxyxanthone	–

[a]Control represents the assay in the absence of the test compounds and the values represent the average of four observations within an error range of 5.0%.

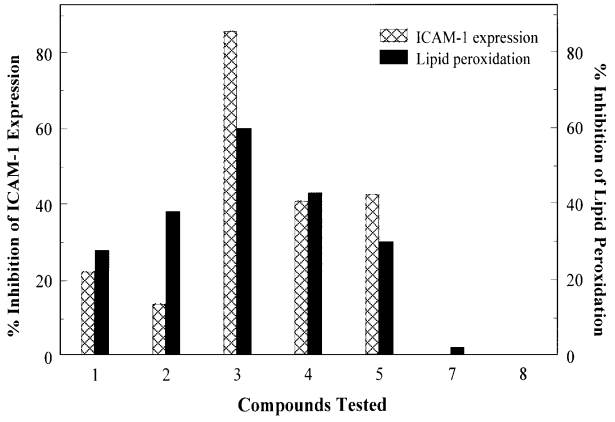

Figure 2. Comparison of inhibition of NADPH catalysed initiation of lipid peroxidation and TNF-α induced ICAM-1 expression on endothelial cells by xanthones.

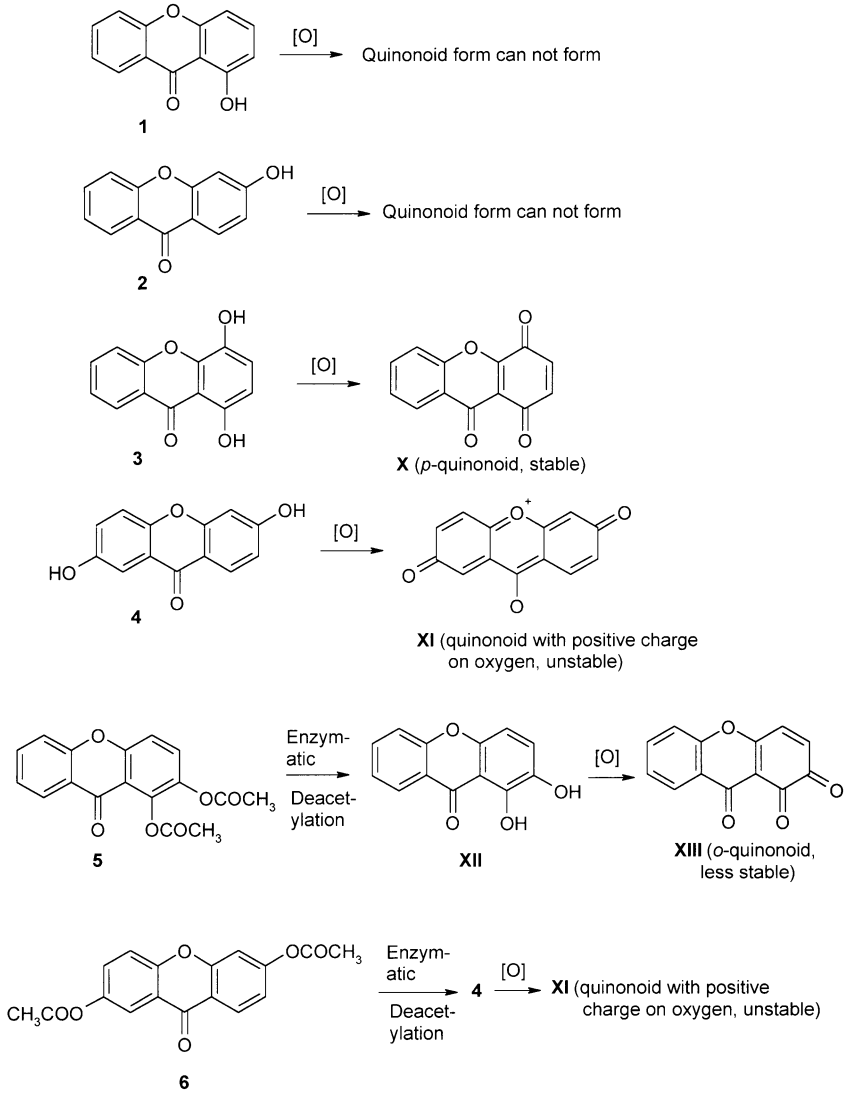

Figure 3. Proposed mechanism of conversion of xanthones to their respective quinonoid forms.

xanthone (**XIII**) from **5** is less than the stability of the *para*-quinonoid xanthone (**X**) from **3**, therefore 1,4-dihydroxyxanthone (**3**) exhibited maximum activity, followed by 1,2-dihydroxy-/1,2-diacetoxyxanthone (**5**), and 2,6-dihydroxyxanthone (**4**) and 2,6-diacetoxyxanthone (**6**). The marked difference in the ICAM-1 expression inhibitory activity of **5** and **6** may be because of the difference in the stability of their corresponding quinonoid forms. The quinonoid form (**XIII**) derived from 1,2-diacetoxyxanthone (**5**) is more stable than the charge separated quinonoid form (**XI**) derived from 2,6-diacetoxyxanthone (**6**) and so the ICAM-1 expression inhibitory activity of compound **5** is almost double than the inhibitory activity of compound **6**. Surprisingly, the xanthone **9**, in spite of having two hydroxy groups does not exhibit any noticeable activity, which may be because of the fact that this compound cannot oxidize, as no quinonoid structure form can be generated. The two methoxyxanthones, **7** and **8** do not exhibit any activity, this may be because of methylation of the free hydroxyl group(s) required for the activity. We propose that xanthones inhibit NADPH-catalyzed liver microsomal lipid peroxidation and ICAM-1 expression on human endothelial cells by getting oxidized to quinones. A similar trend of the two activities (Fig. 2) supports the proposition that hydroxyxanthones, particularly those that can lead to the formation of stable quinonoid structures are more active in the present system of investigation. The generation of such stable quinonoid forms will be confirmed in the future by carrying out quantum chemical calculations and structure optimization studies on these xanthones.

Investigations on several types of polyphenols demonstrated the fact that the phenolic groups and their structures in proximity contributed greatly to their antioxidant potential[12–14] as observed in the present study (Table 2). It is noteworthy that the acetoxyxanthones are quite effective as antioxidants although less compared to the hydroxy derivatives, except 1-hydroxyxanthone. The acetoxypolyphenols are known to be hydrolysed by the non-specific esterases resulting in the generation of phenolic derivatives. Our earlier work has demonstrated that the initiating reactive oxygen radical interacts with the acetoxy group of 7,8-diacetoxy-4-methylcoumarin leading to the formation of phenoxyl radical with the possible loss

of acetyl carbocation.[15] These mechanisms seem to be true in the case of xanthone derivatives (Table 2) also rendering them good antioxidants.

Earlier studies by many groups show that the presence of hydroxyl group on the xanthone nucleus is useful for imparting anti-inflammatory or anti-oxidant activities. For instance, norathyriol (1,3,6,7-tetrahydroxyxanthone), and 1,3- and 3,5-dihydroxyxanthone prevent histamine release from stimulated rat peritoneal mast cells. Norathyriol, 1,3- and 1,6-dihydroxyxanthone, 1,3,7-trihydroxyxanthone, 1,3,5,6-, 2,3,6,7-, and 3,4,5,6-tetrahydroxyxanthones have potent inhibitory effects on superoxide formation by rat neutrophils stimulated with fMLP.[11,16] 1,6-Dihydroxyxanthone and norathyriol also have profound inhibitory effects on hind paw oedema in mice induced by various inflammatory mediators.[10,11] As the formation of reactive oxygen intermediates and activation of cell adhesion molecules is involved in various other pathways involved in inflammation, the results reported here may explain the mechanism underlying the observed activities of xanthones mentioned earlier. Such structure–function relationship studies can help develop better molecules with anti-oxidant and anti-inflammatory activities.

Experimental

Materials

Anti-ICAM-1 antibody and TNF-α were purchased from Parmingen, USA. M199, L-glutamine, penicillin, streptomycin, amphotericin, endothelial cell growth factor, trypsin, Pucks saline, HEPES, DMSO, *o*-phenylenediamine dihydrochloride and anti-mouse IgG-HRP were purchased from Sigma Chemical Co., USA. Fetal calf serum was purchased from Biological Industries, Israel. NADPH, ADP and trichloroacetic acid (TCA) were obtained from Sisco Research Laboratory (Mumbai, India).

Synthesis of xanthones

The xanthones **1**–**9** (Fig. 1) used for the study, that is 1-hydroxyxanthone (**1**),[17] 3-hydroxyxanthone (**2**),[18] 1,4-dihydroxyxanthone (**3**),[17,19] 2,6-dihydroxyxanthone (**4**),[20] 1,2 diacetoxyxanthone (**5**),[21] 2,6-diacetoxyxanthone (**6**),[22] 3-methoxyxanthone (**7**),[23] 1,3,7 trimethoxyxanthone (**8**),[24] and 1,5-dihydroxy-6-methoxyxanthone (**9**)[25] were synthesized using published protocols. They were identified on the basis of comparison of their spectral data and melting points with that reported in the literature.

Cells and cell culture

The primary endothelial cells were isolated from the umbilical cord by mild trypsinisation.[26] Cells were maintained in gelatin-coated tissue culture flasks in M 199 medium supplemented with 20% heat-inactivated fetal calf serum, 2 mM L-glutamine, 100 units/mL penicillin, 100 μg/mL streptomycin, 0.25 μg/mL amphotericin, endothelial cell growth factor (50 μg/mL) and heparin (5 U/mL). The cells were sub-cultured by dislodging with 0.125% trypsin–0.01 M EDTA solution in Pucks saline and HEPES buffer. For the present analysis, cells were used between passages three to four and the viability of cells was determined by trypan blue exclusion test. E-Selectin expression was employed to determine the purity of endothelial cells.

Modified ELISA for measurement of ICAM-1

The expression of ICAM-1 on surface of endothelial cells was quantified using cell-ELISA.[26] Endothelial cells plated to confluence in gelatin coated 96-well plates were incubated with or without xanthones at desired concentrations for 1 h, followed by treatment with TNF-α (10 ng/mL) for 16 h. The cells were fixed with 1.0% glutaraldehyde and non-specific binding of antibody was blocked by using non-fat dry milk (3.0% in PBS). The cells were incubated overnight at 4 °C with ICAM-1 mAb or control IgG Ab (0.25 μg/mL, diluted in blocking buffer), followed by washing with PBS and incubation with peroxidase-conjugated goat anti-mouse secondary Ab (1:1000 diluted in PBS). The cells were again washed with PBS and exposed to the peroxidase substrate (*ortho*-phenylenediamine dihydrochloride 40 mg/100 mL in citrate phosphate buffer, pH 4.5). 2 N sulphuric acid was added to stop the reaction and absorbance at 490 nm was measured using an automated microplate reader (Spectramax 190, Molecular Devices, USA).

Preparation of rat liver microsomes and the assay of initiation of lipid peroxidation

Rat liver microsomes used for the lipid peroxidation studies were prepared adopting the method of Ernster and Nordenbrand.[27] Male rats of wistar strain weighing around 200 g were used for the preparation of liver microsomes. The assay of the initiation of lipid peroxidation has been described previously.[15] Briefly, the reaction mixture consisted of 0.025 M Tris–HCl (pH 7.5), microsomes (1 mg protein), 3 mM ADP and 0.15 mM $FeCl_3$ in a final volume of 2.0 mL. The reaction mixture was incubated at 37 °C for 10 min. To the reaction mixture were then added the test compounds (100 μM each in 0.2 mL DMSO), followed by incubation at 37 °C for 10 min. To the reaction mix was then added 0.5 mM NADPH for the initiation of enzymatic lipid peroxidation and contents incubated for different intervals. The reaction was terminated by the addition of 0.2 mL of 50% TCA, 0.2 mL of 5 N HCl and 1.6 mL of 30% TBA. The tubes were heated in an oil bath at 95C for 30 min, cooled and centrifuged at 3000 rpm. The intensity of the color of the thiobarbituric acid reactive substance (TBRS) formed was measured at 535 nm. The lipid peroxidation was found to be linear upto 15 min under the conditions described here.

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