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Novel thiocoumarins as inhibitors of TNF-α induced ICAM-1 expression on human umbilical vein endothelial cells (HUVECs) and microsomal lipid peroxidation☆,☆☆

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Abstract—Different coumarin/thiocoumarin derivatives, that is, 7-hydroxy-4-methylcoumarin, 7,8-dihydroxy-4-methylcoumarin, 7-acetoxy-4-methylcoumarin, 7,8-diacetoxy-4-methylcoumarin, 7-hydroxy-4-methylthiocoumarin, 7,8-dihydroxy-4-methylthiocoumarin, 7-acetoxy-4-methylthiocoumarin and 7,8-diacetoxy-4-methylthiocoumarin were synthesized and evaluated for their effects on TNF-α induced expression of intercellular adhesion molecule-1 (ICAM-1) on endothelial cells and on NADPH-catalyzed rat liver microsomal lipid peroxidation with a view to identify modulators for expression of cell adhesion molecules and to establish structure–activity relationship. We found that dihydroxy and diacetoxy derivatives of thiocoumarin were more potent in comparison to the corresponding coumarin derivatives in inhibiting TNF-α-induced expression of ICAM-1. However, coumarin derivatives were found to be more potent in comparison to the corresponding thiocoumarins in inhibiting microsomal lipid peroxidation. We have also tested the intermediate compounds 7,8-dibenzyloxy-4-methylcoumarin and 7,8-dibenzyloxy-4-methylthiocoumarin for their inhibitory activity on TNF-α-induced ICMA-1 expression. We found that dibenzyloxy-4-methylthiocoumarin is better than dibenzyloxy-4-methylcoumarin. The mechanisms underlying the observed activities of coumarins and thiocoumarins have been discussed with reference to their structures. Such structure–function relationship studies may help in developing molecules with better anti-inflammatory and anti-oxidant activities.

1. Introduction

The adhesion of leukocytes to the endothelium is among the earliest and essential processes during any inflammatory response. The transendothelial migration of the leukocytes requires an increased expression of cell adhesion molecules on the surface of endothelial cells that interact with their corresponding receptors on the surface of leukocytes.[1] Various inflammatory mediators, for example, cytokines like TNF-α, IL-1β and bacterial lipopolysaccharides increase the expression of cell adhesion molecules, namely intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) and E-selectin on the endothelial cells.[2–5] The increased expression of cell adhesion molecules alters the adhesive property of the vasculature leading to indiscriminate infiltration of the leukocytes across the blood vessels and hence causes inflammation. A regulated expression of cell adhesion molecules is therefore essential for maintaining the body fluidity. Inhibition of cell adhesion molecules has shown to be a useful therapeutic approach to regulate inflammatory response. Monoclonal antibodies (mAbs) specific to cell adhesion molecules and small molecules from natural sources and synthetic

Abbreviations: ICAM-1, Intercellular adhesion molecule-1; TNF-α, Tumor necrosis factor-α; NADPH, Nicotinamide adenine dinucleotide phosphate; NF-κB, Nuclear factor-κB.

Keywords: Coumarins; Thiocoumarins; TNF-α; ICAM-1; Anti-oxidant; Lipid peroxidation.

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routes have been used successfully for downregulating the induced expression of cell adhesion molecules both in vitro and in vivo.[6–8] However, treatment with mAbs is found to be limited because of problems with endotoxin contamination, secondary antibody formation, serum sickness and anaphylaxis.[9]

Coumarins belong to class of polyphenolic compounds that abundantly occur in the plant kingdom. Many compounds of this class are reported to exhibit different biological activities,[10] for example, 4-methylcoumarins have been found to possess choleretic,[11] analgesic,[12] anti-spermatogenic[13] and diuretic properties.[14] 7,8-Dihydroxy-4-methylcoumarin and 7,8-diacetoxy-4-methylcoumarin were reported to exhibit anti-oxidant properties on three counts: (i) efficient scavenging of the oxygen radicals,[15] (ii) prevention of the formation of ADP-perferryl leading to the cessation of the formation of the oxygen radicals[16] and (iii) inhibition of cytochrome P-450-linked mixed function oxidases.[17] The simple coumarins/thiones exhibit hypnotic,[18] hypothermal[19] and insecticidal activities.[20]

Although several activities of coumarins/thiocoumarins have been reported, but not much is known regarding their effects on cytokine-induced cell adhesion molecule expression, which is involved in many inflammatory conditions. Many compounds that inhibit lipid peroxidation and influence the generation of ROS, in turn lead to decrease in the expression of the cell adhesion molecules and subsequently decrease inflammation, hence are found to be useful therapeutic agents in various inflammatory diseases.[21] Treatment of endothelial cells with anti-oxidants is also shown to downregulate the expression of ICAM-1 on endothelial cells.[22] In the present study, for understanding the mechanisms underlying the anti-inflammatory activities of compounds of this class and for establishing structure–activity relationship, we have synthesized several oxygenated coumarins and corresponding thiocoumarins and have studied their inhibitory activities on TNF-α-induced expression of ICAM-1 on HUVECs, and on NADPH-catalyzed liver microsomal lipid peroxidation.

2. Results

7-Hydroxy-4-methylcoumarin (**1**) and 7,8-dihydroxy-4-methylcoumarin (**2**) were synthesized in quantitative yields by Pechmann[23] condensation of resorcinol and pyrogallol, respectively, with ethyl acetoacetate in the presence of concentrated sulfuric acid. The corresponding acetates, that is, 7-acetoxy-4-methylcoumarin (**3**) and 7,8-diacetoxy-4-methylcoumarin (**4**) were synthesized by acetylation of compounds **1** and **2** with acetic anhydride in pyridine in the presence of catalytic amount of dimethylaminopyridine (DMAP) in quantitative yields. The structures of coumarins **1**–**4** were established on the basis of their spectral (^{1}H, ^{13}C NMR and mass) data and comparison of their melting points and spectral data with those reported in the literature.[17,24] Thionation of the coumarins **1** and **2** was attempted by refluxing them in toluene with Lawesson's reagent.[25,26] The thio-analogue **6** of coumarin **1** was obtained in good yield, but because of the poor solubility of coumarin **2** in toluene, its thio-analogue was obtained in very poor yield. To improve the solubility of 7,8-dihydroxy-4-methylcoumarin (**2**), the two hydroxyl groups were protected with benzyl group by refluxing **2** with K_2CO_3 and BnBr in acetone.[27] The dibenzyl derivative **5** was converted to its thio-analogue **7**, which was debenzylated by using $AlCl_3$ and *N*,*N*-dimethylaniline[28] to afford the dihydroxythiocoumarin **8**. The corresponding acetates, 7-acetoxy-4-methylthiocoumarin (**9**) and 7,8-diacetoxy-4-methylthiocoumarin (**10**) were synthesized by the acetylation of **6** and **8**, respectively, by the acetic anhydride–pyridine–DMAP method.

2.1. Coumarin and thiocoumarin derivatives inhibit the TNF-α-induced expression of ICAM-1 on endothelial cells

The effect of 10 different coumarins and thiocoumarins **1**–**10** (Fig. 1) has been examined on the modulation of cytokine-induced expression of ICAM-1 in human endothelial cells (Fig. 2). The endothelial cells plated to confluence in 96 well plates were incubated with varying concentrations of these compounds (Table 1). The effects of these compounds on the viability (determined by trypan blue exclusion test) and the morphology of the endothelial cells (observed under microscope) were also examined. The maximal tolerable concentrations were found to be different for different compounds. For further analysis, the concentrations at the maximal tolerable range were used. The effects of the compounds **1**–**10** on TNF-α-induced ICAM-1 expression were seen using cell-ELISA as detailed in the Experimental Section.

Our results using cell-ELISA demonstrate that ICAM-1 was expressed at low levels on unstimulated endothelial cells and there was over fivefold increase in its expression upon stimulation with TNF-α (data not shown). Pretreatment of endothelial cells with **1**–**10** had no effect on the constitutively expressed levels of ICAM-1 (data not shown), 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 the two thiocoumarins, 7,8-dihydroxy-4-methylthiocoumarin (**8**) and 7,8-diacetoxy-4-methylthiocoumarin (**10**) inhibited the TNF-α induced ICAM-1 expression by 92% and 82%, respectively, at a concentration of 100 μg/mL. The inhibition of ICAM-1 expression by 7,8-dihydroxy-4-methylcoumarin (**2**) and 7,8-diacetoxy-4-methylcoumarin (**4**) at concentrations of 100 and 17.5 μg/mL (maximum tolerable concentrations) was 75% and 60%, respectively, which is less than the corresponding thiocoumarins **8** and **10** (Table 1). The monohydroxy and corresponding monoacetoxy coumarins **1** and **3** and thiocoumarins **6** and **9** did not show appreciable inhibition of TNF-α-induced ICAM-1 expression at the maximum tolerable concentration, viz. 100 μg/mL (Table 1). We have also tested the intermediate compounds 7,8-dibenzyloxy-4-methylcoumarin (**5**) and

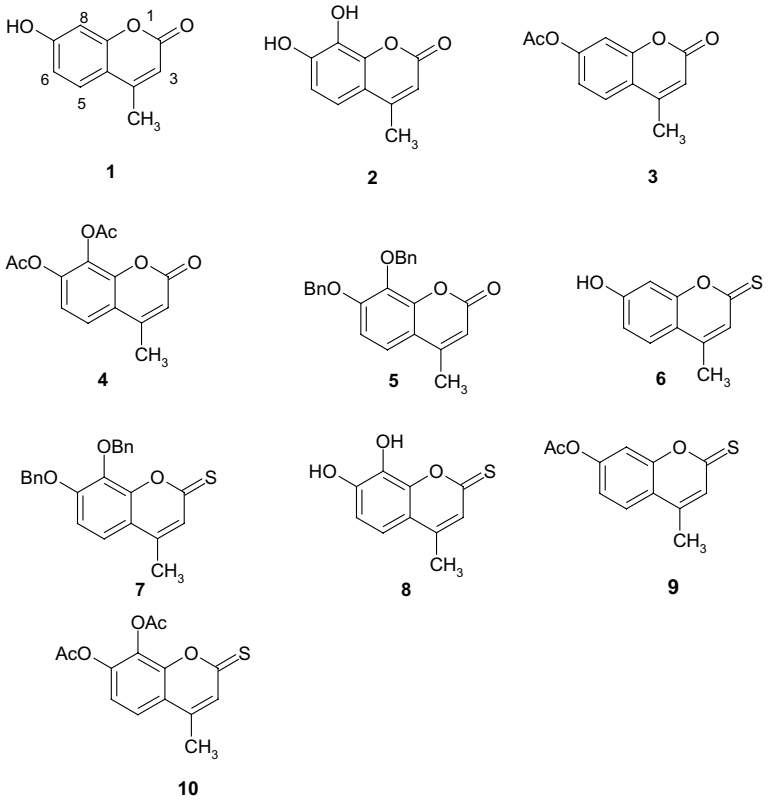

Figure 1. Structures of coumarin and thiocoumarin derivatives.

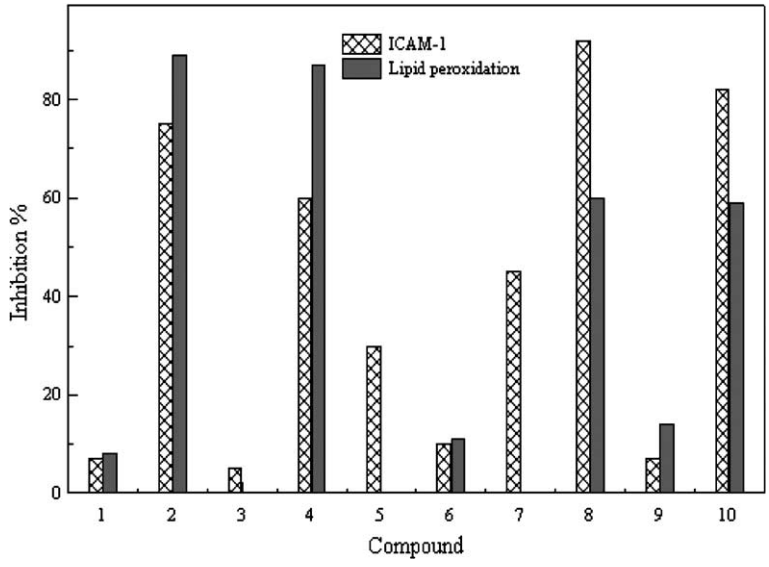

Figure 2. Comparison of inhibition of NADPH catalyzed initiation of lipid peroxidation and TNF-α induced ICAM-1 expression on endothelial cells by coumarins and thiocoumarins.

7,8-dibenzyloxy-4-methylthiocoumarin (**7**) for their inhibitory activity on TNF-α induced ICMA-1 expression. Compound **7** was found to inhibit the ICAM-1 expression by 45%, whereas **5** inhibited the ICAM-1 expression by 30%, thus showing that the intermediate compound dibenzyloxy-4-methylthiocoumarin is better than dibenzyloxy-4-methylcoumarin (Table 1).

2.2. Coumarin and thiocoumarin derivatives inhibit lipid peroxidation

Reactive oxygen species are primary signaling molecules in regulating the expression of ICAM-1 on endothelial cells and hence play an important role in various inflammatory diseases. We have observed that the coumarin

Table 1. Effect of coumarins **1**–**5** and thiocoumarins **6**–**10** on the TNF-α-induced expression of ICAM-1 on endothelial cells

Compounds	Concentration[a]		% Inhibition
	μg/mL	μM	
7-Hydroxy-4-methylcoumarin (**1**)	100	568.2	7
7,8-Dihydroxy-4-methylcoumarin (**2**)	100	520.8	75
7-Acetoxy-4-methylcoumarin (**3**)	100	458.7	5
7,8-Diacetoxy-4-methylcoumarin (**4**)	17.5	63.3	60
7,8-Dibenzyloxy-4-methylcoumarin (**5**)	30	75.9	30
7-Hydroxy-4-methylthiocoumarin (**6**)	100	520.8	10
7,8-Dibenzyloxy-4-methylthiocoumarin (**7**)	30	77.09	45
7,8-Dihydroxy-4-methylthiocoumarin (**8**)	100	478.4	92
7-Acetoxy-4-methylthiocoumarin (**9**)	100	427.3	7
7,8-Diacetoxy-4-methylthiocoumarin (**10**)	100	341.2	82

The data presented are representative of three independent experiments. Values shown are mean ± SD of quadruplicate wells.
[a] The concentration levels of different compounds are based on their maximum tolerable concentration by the cells.

and thiocoumarin derivatives **1**–**10** inhibit ICAM-1 expression, while some of these compounds viz. **1**–**4**, **6** and **8**–**10** also inhibit lipid peroxidation.

We have already reported the inhibitory effects of coumarin derivatives **2**–**4** on lipid peroxidation.[15] It has been found that 7,8-dihydroxy-4-methylcoumarin (**2**) and 7,8-diacetoxy-4-methylcoumarin (**4**) profoundly inhibit the rat liver microsomal lipid peroxidation (Table 2). The monohydroxycoumarin **1** and its acetyl derivative **3** did not have any significant microsomal lipid peroxidation inhibitory activity. Herein we have examined the effect of thiocoumarins **6**, **8**, **9** and **10** on initiation of lipid peroxidation in rat liver microsomes. The results presented in Table 2 illustrate the influence of these compounds on the enzymatic initiation of lipid peroxidation. Again as in the case of coumarins, the monohydroxy 4-methylthiocoumarin **6** and monoacetoxy 4-methylthiocoumarin **9** were found to exhibit insignificant inhibition of initiation of lipid peroxidation in rat liver microsomes. However, the other two thiocoumarins, that is, 7,8-dihydroxy-4-methylthiocoumarin (**8**) and 7,8-diacetoxy-4-methylthiocoumarin (**10**) exhibited much effective inhibition of lipid peroxidation in rat liver microsomes, both these compounds inhibited rat liver microsomal lipid peroxidation initiated enzymatically by the addition of NADPH to the reaction mixture to an extent of 60% (Table 2).

Efforts were made to evaluate the activity of 7,8-dihydroxy-4-methylthiocoumarin (**8**) and 7,8-diacetoxy-4-methylthiocoumarin (**10**) to terminate the lipid peroxidation. The compounds were added to the reaction mixture of lipid peroxidation at different time intervals after initiation of the reaction (Table 3). 7,8-Dihydroxy- and 7,8-diacetoxy-4-methylthiocoumarins **8** and **10** were found to terminate the lipid peroxidation up to an extent of 47% and 45%, respectively, when added after 30 min of initiation of lipid peroxidation. This simply demonstrated the excellent anti-oxidant activity of dihydroxy- and diactetoxy-4-methylthiocoumarins.

The radical scavenging abilities of 4-methylthiocoumarin derivatives **6**, **8**, **9** and **10** based on the determination of drop in the absorption of stable radical of DPPH (1,1-diphenyl-1-picrylhydrazyl) have been determined and the results are documented in Table 4; dihydroxy-4-methylthiocoumarin (**8**) scavenged DPPH to the extent of 99.97% at a concentration of 100 μM. The

Table 3. Termination of lipid peroxidation initiation by 4-methylthiocoumarins

Compound	Time (min)			
	5	10	15	30
6	3.50	6.94	9.75	11.10
8	13.21	26.73	37.23	46.79
9	3.23	5.57	9.25	10.75
10	13.51	25.67	39.61	44.66

The data presented are representative of three independent experiments.

Table 2. Effect of 4-methylcoumarin and thiocoumarin derivatives (at 100 μM concentration) on NADPH-catalyzed rat liver microsomal lipid peroxidation initiation

Compound	% Inhibition of NADPH-catalyzed lipid peroxidation	Ratio ICAM-1/lipid peroxidation
7-Hydroxy-4-methylcoumarin (**1**)	8	0.875
7,8-Dihydroxy-4-methylcoumarin (**2**)	89[a]	0.843
7-Acetoxy-4-methylcoumarin (**3**)	0[a]	0
7,8-Diacetoxy-4-methylcoumarin (**4**)	87[a]	0.689
7-Hydroxy-4-methylthiocoumarin (**6**)	11	0.909
7,8-Dihydroxy-4-methylthiocoumarin (**8**)	60	1.53
7-Acetoxy-4-methylthiocoumarin (**9**)	14	0.5
7,8-Diacetoxy-4-methylthiocoumarin(**10**)	59	1.39

The data presented are representative of three independent experiments.
[a] This data has been reported by us previously.[15]

Table 4. Radical scavenging potential of 4-methylthiocoumarins at 100 μM concentration

Compound	Scavenging of DPPH (in %)[a]
7-Hydroxy-4-methylthiocoumarin (**6**)	23.28
7,8-Dihydroxy-4-methylthiocoumarin (**8**)	99.97
7-Acetoxy-4-methylthiocoumarin (**9**)	14.38
7,8-Diacetoxy-4-methylthiocoumarin (**10**)	47.51

The data presented are representative of three independent experiments.

[a] The decolourization of DPPH by various test compounds was carried out as described in Experimental Section.

radical scavenging activity of 7,8-diacetoxy-4-methylthiocoumarin (**10**) was found to be almost half of the activity of thiocoumarin **8**, 7-hydroxy- and 7-acetoxy-4-methylthiocoumarins (**6**) and (**9**) did not exhibit appreciable activity at a concentration of 100 μM.

It has been observed that dihydroxy and diacetoxy thiocoumarins **8** and **10** were more potent in comparison to the corresponding coumarin analogues **2** and **4** in inhibiting TNF-α induced expression of ICAM-1. However, coumarins **2** and **4** were found to be more potent in comparison to the thiocoumarins **8** and **10** in inhibiting microsomal lipid peroxidation. In separate set of experiments, we have observed that thiocoumarin acetates were more potent in inhibiting protein kinase C (PKC) catalyzed by the purified rat liver microsomal transacetylase (TAase) (unpublished data). Also, thiocoumarin acetates exhibited high specificities with TAsae and hence highly effective in causing TAase mediated biological action (unpublished data). As PKC is associated in the activation of TNF-α induced ICAM-1 expression, thiocoumarins could be more potent in inhibiting ICAM-1 expression than their corresponding coumarin derivatives. The monohydroxy and monoacetoxy coumarins **1** and **3**, and thiocoumarins **6** and **9** did not exhibit appreciable activity either for the inhibition of TNF-α-induced expression of ICAM-1 or for the inhibition of NADPH-catalyzed rat liver microsomal lipid peroxidation. Although the inhibition patterns of NADPH-catalyzed lipid peroxidation and TNF-α-induced ICAM-1 expression follow very similar trend, however, we have noted that dihydroxy- and diacetoxythiocoumarins **8** and **10** are more potent in inhibiting TNF-α-induced ICAM-1 expression (Fig. 2).

3. Discussion

In the present investigation, coumarins **1**–**5** and thiocoumarins **6**–**10** have been evaluated for their ability to modulate TNF-α induced ICAM-1 expression and for the inhibition and termination of initiation and propagation steps of NADPH-catalyzed microsomal lipid peroxidation, respectively, in order to examine their anti-oxidant property. As shown in Tables 1 and 2, hydroxy substituent on the coumarin and thiocoumarin nucleus is required for both, anti-oxidant activity and ICAM-1 inhibitory activity. For example, dihydroxy compounds showed high inhibitory activity as compared to monohydroxy compounds in both, the coumarin and thiocoumarin series. This indicates that increase in the number of hydroxy groups on the coumarin or thiocoumarin nucleus enhances the anti-oxidant as well as ICAM-1 inhibitory activity. The activity of dihydroxy and diacetoxy coumarin and thiocoumarin derivatives 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 dihydroxythiocoumarins have better activity as compared to diacetoxythiocoumarins, this may be due to the higher rate constant for the formation of phenoxyl redical by hydroxy derivatives as compare to acetoxy derivatives. We propose that these compounds 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 hydroxycoumarins and hydroxythiocoumarins, particularly those that can lead to the formation of stable quinonoid structures are more active in the present system of investigation. We have also tested the intermediate compounds 7,8-dibenzyloxy-4-methylocoumarin (**5**) and 7,8-dibenzyloxy-4-methylthiocoumarin (**7**) and found that comparatively they show much less activity. The possible reason for their less activity may be that they are not capable of forming the stable quinonoid forms as the benzyl moieties, being joined through ether linkages cannot be converted into free phenolic analogues in the animal systems (Fig. 3). In Figure 3, acetyl derivatives first transformed to hydroxy derivatives before forming the stable quinonoid form.[29] Investigations by several groups of researchers on polyphenolic compounds demonstrated the fact that the presence of phenolic groups contributes greatly to the anti-oxidant potential of the compounds[30–32] as observed by us in present study (Table 2). It is noteworthy that dihydroxy compounds are more effective as compared to diacetoxy compounds.

Our earlier work has demonstrated that the initiating reactive oxygen radical interacts with the acetoxy group of 7,8-diacetoxy-4-methylcoumarin (**4**) leading to the formation of phenoxy radical with the possible loss of acetyl carbocation.[33] This mechanism seems to be true in the case of thiocoumarin derivatives as well and thereby renders them good anti-oxidants. Recent studies suggest that increased circulating lipid peroxides in pre-eclemptic women are responsible for increased ICAM-1 expression on endothelial cells,[34] so the compounds which can inhibit the lipid peroxidation, could also inhibit the ICAM-1 expression on endothelial cells,[35] our results support the earlier observations. 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 coumarins and thiocoumarins. Such structure–function relationship studies can help in developing better molecules with anti-oxidant and anti-inflammatory activities.

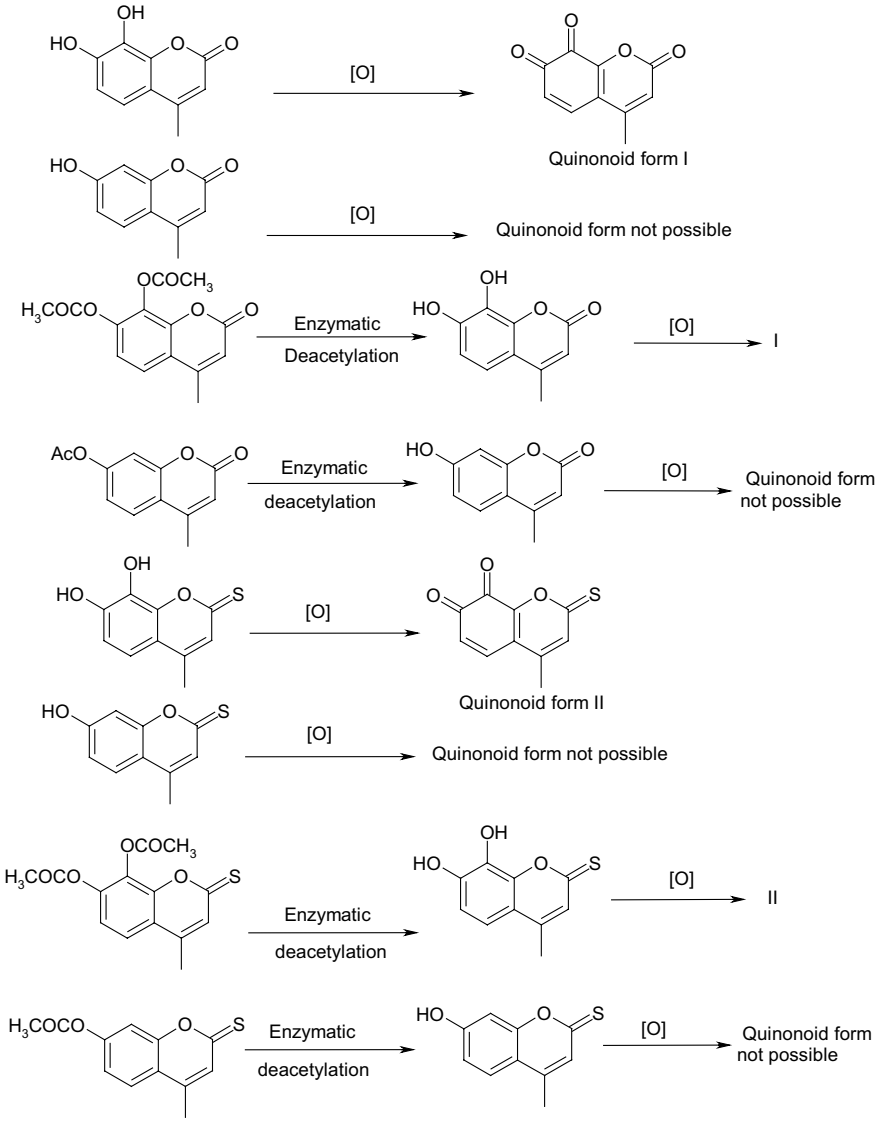

Figure 3. Proposed mechanism of conversion of coumarins and thiocoumarins to their respective quinonoid forms.

4. Experimental section

4.1. Chemicals

The organic solvents (acetone, toluene, CH_2Cl_2, pyridine) were dried and distilled prior to their use. Analytical TLCs were performed on precoated Merck silica gel 60 F_{254} plates; the spots were visualized under UV light. Melting points were determined in a sulfuric acid bath and are uncorrected. The IR spectra were recorded on a Perkin–Elmer model 2000 FT-IR spectrophotometer. The ^1H NMR and ^{13}C NMR spectra were recorded on a Bruker Avance instrument at 300 and 75.5 MHz, respectively, using TMS as internal standard. The chemical shift values are on δ scale and the coupling constant values (J) are in Hz. The HRMS were recorded on a TMS-AX 505 W instrument. Anti-ICAM-1 antibody and TNF-α were purchased from Pharmingen, 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. Foetal calf serum was purchased from Biological Industries, Israel. NADPH, ADP and trichloroacetic acid (TCA) were obtained from Sisco Research Laboratory (Mumbai, India).

4.2. 7,8-Dibenzyloxy-4-methylcoumarin (5)

To a mixture of 7,8-dihydroxy-4-methylcoumarin (**2**) (8 g, 42.0 mmol) and benzyl bromide (15.7 g, 10.9 mL) in dry acetone (100 mL) was added anhydrous K_2CO_3 (17.3 g) and the reaction mixture was refluxed for 10 h. The progress of the reaction was monitored by TLC. On completion, the solvent was removed under vacuum and the residue was poured into ice-cold water (75 mL). The aqueous reaction mixture was extracted with ethyl acetate (3×50 mL) and the combined organic layer was dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. The residue, thus obtained was washed with petroleum ether and purified by column chromatography on silica gel using gradient solvent

system of ethyl acetate and petroleum ether to afford 7,8-dibenzyloxy-4-methylcoumarin (**5**) as a white solid (13.0 g) in 84% yield, mp 152 °C; R_f: 0.46 (petroleum ether/ethyl acetate, 4:1); IR (KBr): 2925, 2361, 1712 (C=O), 1607, 1293, 1088, 981 and 697 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz): δ 2.35 (3H, s, CH_3), 5.17 and 5.19 (4H, 2s, 2H each, $2\times OCH_2C_6H_5$), 6.13 (1H, s, C-3H), 6.90 (1H, d, J = 8.9 Hz, C-6H), 7.21–7.51 (11H, m, C-5H and $2\times OCH_2C_6H_5$); ^{13}C NMR ($CDCl_3$, 75.5 MHz): 20.01 (CH_3), 72.63 and 76.94 ($2\times O–CH_2–C_6H_5$), 111.38 (C-6), 113.95 (C-3), 116.43 (C-10), 120.79 (C-5), 128.69, 129.40, 129.52, 129.55, 129.97 ($2\times OCH_2C_6H_5$), 137.56 and 138.42 (C-7 and C-8), 153.7 (C-4), 156.06 (C-9) and 161.78 (C-2). HRMS calcd for $C_{24}H_{20}O_4$ $[M+Na]^+$ 395.1254, found 395.1243.

4.3. General procedure for the thionation of coumarins 1 and 5

A mixture of coumarin **1** or **5** (5 g, 28.4 or 13.4 mmol) and Lawesson's reagent[25,26] (5.7 or 2.71 g, 14.2–6.7 mmol) was refluxed in toluene (40 mL) for 24 h. The progress of the reaction was monitored by TLC. On completion, the reaction mixture was allowed to cool to room temperature and the solvent evaporated to dryness in vacuum, the residue was purified by column chromatography on silica gel using gradient solvent system of ethyl acetate–petroleum ether to afford 7-hydroxy-4-methylthiocoumarin (**6**) and 7,8-dibenzyloxy-4-methylthiocoumarin (**7**) as yellow solids in 44% and 29% yields, respectively. 7-Hydroxy-4-methylthiocoumarin (**6**) was identified on the basis of the spectroscopic data, which was found to be identical with the spectroscopic data reported in the literature.[36]

4.4. 7,8-Dibenzyloxy-4-methylthiocoumarin (7)

It was obtained as a yellow solid (1.5 g) in 29% yield, mp 130–135 °C; R_f: (petroleum ether/ethyl acetate, 4:1); IR (KBr): 2924, 1596, 1378, 1103, 965 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz): δ 2.27 (3H, s, CH_3), 5.19 and 5.25 (4H, 2s, 2H each, $2\times OCH_2C_6H_5$) and 6.92–7.54 (13H, m, C-3H, C-5H, C-6H and $2\times OCH_2C_6H_5$); ^{13}C NMR ($CDCl_3$, 75.5 MHz): 18.39 (CH_3), 71.77 and 76.20 ($2\times O–CH_2–C_6H_5$), 111.74 (C-6), 116.99 (C-10), 119.77 (C-5), 127.40, 127.73, 128.55, 128.64, 129.07, 129.27 (C-3, $2\times O–CH_2–C_6H_5$), 136.45, 137.26 (C-7, C-8), 144.61 (C-4), 155.29 (C-9), 197.16 (C-2); HRMS calcd for $C_{24}H_{21}O_3S$ $[M+H]^+$ 389.1211, found 389.1221.

4.5. Synthesis of 7,8-Dihydroxy-4-methylthiocoumarin (8)

To a solution of the thiocoumarin **7** (1.0 g, 2.58 mmol) and *N*,*N*-dimethylaniline (1.25 g, 10.32 mmol) in CH_2Cl_2 (20 mL), powdered $AlCl_3$ (1.03 g, 7.74 mmol) was added in portions. The reaction mixture was stirred at room temperature for 45 min and on completion, the reaction was quenched by the addition of 1 N HCl (4.5 mL). The aqueous reaction mixture was extracted with ethyl acetate (3×30 mL), combined organic layer was washed with 5% $NaHCO_3$ solution, dried over anhydrous Na_2SO_4 and the solvent concentrated under reduced pressure. The residue, thus obtained was purified by column chromatography using gradient system of ethyl acetate–petroleum ether to afford 7,8-dihydroxy-4-methylthiocoumarin (**8**) as a yellow solid (0.30 g) in 56% yield, mp 230–235 °C; R_f: 0.48 (petroleum ether/ethyl acetate, 3:2); IR (KBr): 3312 (OH), 2925, 2855, 1561, 1434, 1366, 1254 and 1095 cm^{-1}; ^1H NMR (DMSO-d_6 + $CDCl_3$, 300 MHz): δ 2.34 (3H, s, CH_3), 6.95 (1H, d, J = 8.6Hz, C-6H), 7.02 (1H, s, C-3H), 7.09 (1H, d, J = 8.6 Hz, C-5H), 8.83 and 8.94 (2H, 2s, 1H each, $2\times OH$); ^{13}C NMR (DMSO-d_6 + $CDCl_3$, 75.5 MHz): δ 20.68 (CH_3), 116.42 (C-6), 117.72 (C-10), 118.07 (C-5), 118.31 (C-3), 134.23 (C-4), 148.55 and 148.93 (C-7 and C-8), 151.96 (C-9) and 199.31 (C-2); HRMS calcd for $C_{10}H_9O_3S$ $[M+H]^+$ 209.0272, found 209.0260.

4.6. General procedure for the acetylation of thiocoumarins 6 and 8

To a solution of the thiocoumarin **6** or **8** (0.2 g, 1.04 or 0.96 mmol) in acetic anhydride (1.1 equiv) and pyridine (2 equiv) was added a catalytic amount of DMAP and the reaction mixture was stirred at 25–28 °C for 6–8 h. The progress of the reaction was monitored by TLC. After completion, the reaction mixture was poured into ice-cold water, the solid that precipitated was filtered and washed with petroleum ether, dried and recrystallized from $CHCl_3$ to afford the corresponding acetates **9** and **10** in 54 or 83% yields, respectively. The structure of monoacetoxycoumarin **9** was established on the basis of physical and spectral data analysis and by comparing it with the data reported in the literature.[36]

4.7. 7,8-Diacetoxy-4-methylthiocoumarin (10)

It was obtained as a yellow solid (0.15 g) in 83% yield, mp 190–195 °C; R_f: 0.52 (petroleum ether/ethyl acetate, 2:1); IR (KBr): 2925, 2854, 2361, 1774 (C=O), 1562, 1431, 1376, 1292, 1163, 1096, 1015 and 873 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz): δ 2.34 and 2.36 (6H, 2s, 3H each, $2\times OCOCH_3$), δ 2.43 (3H, s, CH_3), 7.12 (1H, s, C-3H), 7.20 (1H, d, J = 8.5Hz, C-5H) and 7.50 (1H, d, J = 8.5 Hz, C-6H); ^{13}C NMR ($CDCl_3$, 75.5 MHz): δ 18.36 (CH_3), 20.65 and 21.00 ($2\times OCOCH_3$), 120.04 (C-6), 120.82 (C-10), 121.79 (C-5), 129.47 (C-3), 143.16 (C-7 and C-8), 145.77 (C-4 and C-9), 167.75 and 167.93 ($2\times C=O$) and 196.15 (C=S); HRMS calcd for $C_{14}H_{13}O_5S$ $[M+H]^+$ 293.0484, found 293.0514.

4.8. Cells and cell culture

The primary endothelial cells were isolated from the umbilical cord by mild trypsinisation.[8] Cells were maintained in gelatin coated tissue culture flasks in M 199 medium supplemented with 20% heat inactivated foetal 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.

4.9. Modified ELISA for measurement of ICAM-1

The expression of ICAM-1 on surface of endothelial cells was quantified using cell-ELISA.[8] Endothelial cells plated to confluence in gelatin coated 96 well plates were incubated with or without compounds 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 nonspecific binding of antibody was blocked by using non-fat dry milk (3.0% in PBS). The cells were incubated overnight at 40 °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 sulfuric acid was added to stop the reaction and absorbance at 490 nm was measured using an automated microplate reader (Spectramax 190, Molecular Devices, USA).

4.10. 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.[37] 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.[26] Briefly, the reaction mixture consisted of Tris–HCl (0.025 M, pH 7.5), microsomes (1 mg protein), ADP (3 mM) and $FeCl_3$ (0.15 mM) 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 mixture was then added NADPH (0.5 mM) for the initiation of enzymatic lipid peroxidation and contents incubated for different intervals. The reaction was terminated by the addition 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 95 °C for 30 min, cooled and centrifuged at 3000 rpm. The intensity of the colour 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.

4.11. The assay of chain termination lipid peroxidation

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 30 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 the intervals of 5, 10, 15 and 30 min.

4.12. Assay of DPPH radical scavenging

A solution of test compounds in methanol (4 mL) at concentration of 100 μM was added to 1 mL of DPPH solution in methanol (0.15 mM). The contents were vigorously mixed and allowed to stand at 20 °C for 30 min and the absorbance was taken at 517 nm.

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