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A chromone analog inhibits TNF-α induced expression of cell adhesion molecules on human endothelial cells via blocking NF-κB activation☆

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Abstract—The interaction between leukocytes and the vascular endothelial cells (EC) via cellular adhesion molecules plays an important role in various inflammatory and immune diseases. The molecules that block these interactions have been targeted as potential therapeutic targets for acute and chronic inflammatory diseases. In an effort to develop potent cell adhesion molecule inhibitors, a series of chromone derivatives bearing alkoxycarbonylvinyl unit at the C-3 position, that is, the chromones **8a–d** and **9a–d**, were designed and synthesized, and evaluated for their ICAM-1 inhibitory activity on human endothelial cells as well as their effect on NADPH-catalyzed rat microsomal lipid peroxidation. A structure–activity relationship was established and we found that length of the alkyl moiety of the chromone-3-yl-acrylate is important for this activity. Further, we found that incorporation of unsaturation in the alcohol moiety increases the potential of the compound for the inhibition of TNF-α induced expression of ICAM-1 and also for the inhibition of lipid peroxidation. Out of the screened compounds, the most potent compound ethyl *trans*-3-(4-oxo-4*H*-1-benzopyran-3-yl)-acrylate (**8a**) was taken for further study. We have found that compound **8a** also significantly inhibited the TNF-α induced expression of VCAM-1 and E-selectin, which play key roles in various inflammatory diseases. This inhibition was found to be concentration dependent. The functional consequences of inhibiting cell adhesion molecules were studied by performing cell-adhesion assay. We found that compound **8a** significantly blocks the adhesion of neutrophils to endothelial monolayer. To elucidate the molecular mechanism of inhibition of cell adhesion molecules, we investigated the status of nuclear transcription factor-κB (NF-κB) and were able to establish that compound **8a** significantly blocked the TNF-α induced activation of NF-κB.

1. Introduction

The recruitment of leukocytes from the blood into tissue is central to the development and maintenance of a majority of inflammatory diseases. This multistep process requires a series of leukocyte-endothelial adhesive interactions, involving several families of adhesion molecules.[1] Adhesion molecules participate in several inflammatory reactions mainly by regulation of leukocyte migration, activation, and survival. The elevated expression of the cell adhesion molecules, such as

Abbreviations: CAMs, cell adhesion molecules; ICAM-1, intercellular adhesion molecule-1; VCAM-1, vascular cell adhesion molecule-1; TNF-α, tumor necrosis factor-α; NF-κB, nuclear factor-κB; EMSA, electrophoretic mobility shift assay; HUVECs, human umbilical cord vein endothelial cells.

Keywords: Chromones; Endothelial cells; TNF-α; CAMs; ICAM-1; NF-κB.

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ICAM-1, VCAM-1, and E-selectin, on the luminal surface of vascular endothelial cells is a critical early event in inflammatory processes.[2–5] The molecules that block these interactions have been targeted as potential therapeutic treatments for acute and chronic inflammatory diseases.

Several anti-adhesion therapies, like use of specific monoclonal antibodies (mAbs), have been found to be beneficial for controlling various diseases.[6] However, due to endotoxin contamination, unpredictable clinical manifestations such as secondary antibody formation, cellular activation, and other complications like serum sickness and anaphylaxis, the practical use of mAbs is limited.[7] Furthermore, it is crucial to understand the underlying mechanisms of leukocyte recruitment in affected tissues and for developing effective anti-adhesion therapy. Recently, many groups including our laboratory have identified a number of small molecules from natural/synthetic sources as well as several plant extracts that block nuclear accumulation of NF-κB and abrogate TNF-α induced expression of E-selectin, VCAM-1, and ICAM-1 on endothelial cells.[8–14] The efficacies of some of the identified compounds have also been tested using in vivo models.[15–18] Similarly antioxidants and proteasome inhibitors can suppress this regulatory system.[19] Inhibition of these molecules by various small molecules has been shown to downregulate the expression of cell adhesion molecules and is effective in controlling various inflammatory diseases.[20]

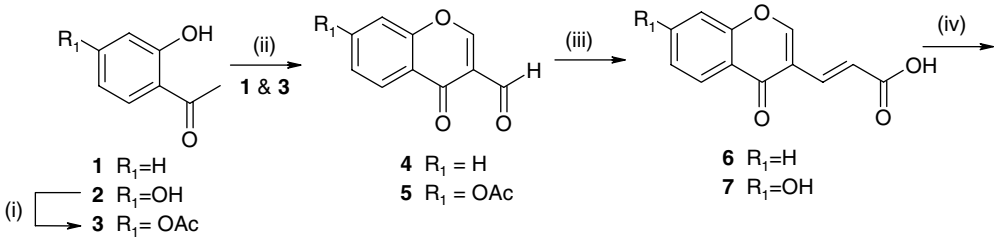
Chromones constitute one of the major classes of naturally occurring compounds. Chromones and their structural analogs are of great interest because of their usefulness as biologically active agents.[21] Due to their abundance in plants and their low mammalian toxicity, chromone derivatives are present in large amounts in the diet of humans.[22,23] Some of the biological activities attributed to chromone derivatives include cytotoxic (anticancer),[24–26] neuroprotective,[27] HIV-inhibitory,[28] antimicrobial,[29,30] antifungal,[31] and antioxidant activity.[32] Chromones are thought to act by stabilizing the mast cell membrane, thus inhibiting mediator release in asthma. These compounds also protect against allergen induced increment in airway hyperreactivity in experimental animals.[33] Although chromones have great pharmaceutical importance, no study has yet been done to find their effect on cellular trafficking in inflammatory conditions by modulating the expression of cell adhesion molecules.

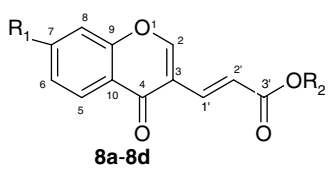
In the present study, we report the design, synthesis, and inhibitory activity of chromone derivatives as inhibitors of TNF-α induced expression of ICAM-1 on HUVECs. Also the structure–activity relationship of various chromones has been studied. In an attempt to elucidate the mechanism underlying the observed activity, we have demonstrated that chromone derivative **8a** is capable of inhibiting TNF-α induced activation of NF-κB. The implication of this study in developing better anti-inflammatory molecules has been discussed.

2. Results

2.1. Chemistry

The compounds described in this study were prepared by following a straightforward procedure depicted in Scheme 1. The benzopyranonyl aldehydes **4** and **5** were obtained by the reaction of acetophenones **1** and **3**, respectively, with dimethylformamide and $POCl_3$, which were further converted into benzopyranonyl acrylic acids **6** and **7**[34–39] by condensation with malonic acid.





Reagents and conditions:
(i) Ac_2O, pyr, 30 °C, 2h.
(ii) $POCl_3$, anhyd. DMF, 55-60 °C, 13 h.
(iii) $CH_2(COOH)_2$, pyr, reflux.
(iv) C_2H_5OH/*n*-C_3H_7OH/CH_2=CH-CH_2OH/$(CH_3)_2CH$-OH, concentrated H_2SO_4, reflux.

Compd.	a	b	c	d
8	R_1=H, R_2=C_2H_5	R_1=H, R_2=*n*-C_3H_7	R_1=H, R_2=C_3H_5	R_1=H, R_2=*iso*-C_3H_7
9	R_1=OH, R_2=C_2H_5	R_1=OH, R_2=*n*-C_3H_7	R_1=OH, R_2=C_3H_5	R_1=OH, R_2=*iso*-C_3H_7

Scheme 1. Systematic pathway for synthesis of chromone derivatives.

Table 1. Effect of *trans*-3-(4-oxo-4*H*-1-benzopyran-3-yl)-acrylates **8a–d** and **9a–d** and chromone on TNF-α induced expression of ICAM-1 on endothelial cells[a]

Compound	Inhibition (%)	Concentrations		IC_{50}		% Cells viability
		μg/ml[b]	μM	μg/ml	μM	
DMSO (0.25%)	—	0.25%	—	—	—	97
Ethyl *trans*-3-(4-oxo-4*H*-1-benzopyran-3-yl)-acrylate (**8a**)	90 ± 5.09	90	368	45	184	96
n-Propyl *trans*-3-(4-oxo-4*H*-1-benzopyran-3-yl)-acrylate (**8b**)	81.75 ± 1.7	80	330	55	227	97
Allyl *trans*-3-(4-oxo-4*H*-1-benzopyran-3-yl)-acrylate (**8c**)	64.5 ± 5.3	90	348	65	251	95
iso-Propyl *trans*-3-(4-oxo-4*H*-1-benzopyran-3-yl)-acrylate (**8d**)	73.5 ± 6.0	70	271	60	232	98
Ethyl *trans*-3-(7-hydroxy-4-oxo-4*H*-1-benzopyran-3-yl)-acrylate (**9a**)	68.25 ± 12.5	90	346	70	269	95
n-Propyl *trans*-3-(7-hydroxy-4-oxo-4*H*-1-benzopyran-3-yl)-acrylate (**9b**)	63.5 ± 7.0	110	426	90	349	97
Allyl *trans*-3-(7-hydroxy-4-oxo-4*H*-1-benzopyran-3-yl)-acrylate (**9c**)	55.25 ± 11.6	100	364	75	273	97
iso-Propyl *trans*-3-(7-hydroxy-4-oxo-4*H*-1-benzopyran-3-yl)-acrylate (**9d**)	52.5 ± 5.8	60	218	55	218	96
Chromone	47.55 ± 4.9	90	616	—	—	95

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

The acrylic acid analogs **6** and **7** were freshly recrystallized prior to esterification by reacting with corresponding alcohol (ethyl alcohol, *n*-propyl alcohol, allyl alcohol, and isopropyl alcohol) under acidic condition to the form acrylates **8a–d** and **9a–d**. All the intermediates **4–7** and final acrylates **8a–d** and **9a–d** were unambiguously characterized on the basis of their spectral data (^1H, ^{13}C NMR, IR, UV, EIMS, HRMS). Compounds **8b–d** and **9a–d** are novel and are being reported for the first time. The structures of known compounds including chromone were further confirmed by comparing their mp/spectral data with those reported in the literature.[34,40,41]

2.2. Chromones are not toxic to cells

The chromone analogs **8a–d** and **9a–d** were examined for their cytotoxic effect on human endothelial cells as described in Section 4, and were not toxic to endothelial cells as more than 95% cells were viable at maximum tolerable concentration (Table 1). This implied that chromone derivatives are safe to use at indicated concentrations. The maximal tolerable concentrations were found to be different for different compounds. For all further analyses, the maximal tolerable concentrations were used.

2.3. Chromone derivatives inhibit TNF-α induced expression of ICAM-1

The effect of chromone and its various analogs **8a–d** and **9a–d** on TNF-α induced expression of ICAM-1 was examined on endothelial cells as described in Section 4. Briefly, HUVECs were incubated with or without these derivatives at various concentrations for 2 h prior to induction with TNF-α (10 ng/ml) for 16 h for ICAM-1 expression. As detected by cell-ELISA, ICAM-1 was expressed at low levels on unstimulated endothelial cells and was induced almost 3-fold by stimulation with TNF-α (data not shown). The chromone derivatives inhibited the TNF-α induced expression of ICAM-1 to different extents. As the maximum tolerable concentrations used in these experiments are to some degree different, a direct comparison cannot be made, thus the IC_{50} values of all the compounds were calculated separately from their respective activity–concentration graphs (Table 1). Also, the maximum levels of inhibition (%) have been shown at the maximum tolerable concentration where cell viability and morphology were not affected by the tested compounds (Table 1). The screening data in Table 1 revealed that ethyl *trans*-3-(4-oxo-4*H*-1-benzopyran-3-yl)-acrylate (**8a**) is the most active compound among all the eight chromone-3-yl-acrylates **8a–d** and **9a–d** evaluated for inhibition of TNF-α induced expression of ICAM-1. Compound **8a** exhibited 90% inhibition of TNF-α induced expression of ICAM-1; the IC_{50} value of the compound is 45 μg/ml, which is also lowest among all the tested chromones. The change of alcohol moiety of chromone-3-yl-acrylate from ethyl to propyl reduces the inhibitory activity; thus the activity of propyl chromone-3-yl-acrylate **8b** is about 1.4 times less than the activity of ethyl chromone-3-yl-acrylate **8a**. Introduction of unsaturation in the alcohol moiety increases the potential of the compound for the inhibition of TNF-α induced expression of ICAM-1. Thus, although propyl chromone-3-yl-acrylate **8b** and allyl chromone-3-yl-acrylate **8c** have the alcohol moiety with the same number of carbon atoms, compound **8c** is 1.3 times more active than compound **8b**, and the former compound has unsaturated alcohol moiety. However, the activity of inhibition of TNF-α induced expression of ICAM-1 by ethyl chromone-3-yl-acrylate **8a** with two carbon-alcohol moiety is still higher than that of allyl chromone-3-yl-acrylate **8c** with the three carbon-unsaturated alcohol moiety. IC_{50} value of compound **8c** is higher as compared to that of **8a**. *Iso*-propyl chromone-3-yl-acrylate **8d**, although has alcohol moiety with the same number of carbon atoms as the compound **8b**, still **8b** is more active than the latter compound, **8d**. This indicates that different arrangement of same number of carbon atoms in the alcohol moiety affects the potential of chromone-3-yl-acrylates as inhibitors of TNF-α induced expression of ICAM-1. The data in Table 1 suggest that introduction of hydroxyl group in the aromatic ring of chromone-3-yl-acrylates decreases the potential of the compounds as inhibitors of the TNF-α induced expression of ICAM-1. Thus TNF-α induced expression of ICAM-1 inhibitory activity of 7-hydroxychromone-3-yl-acrylates **9a–d**, in general, is less than the activities of chromone-3-yl-acrylates **8a–d** without

hydroxyl group in the aromatic moiety. The trend of inhibition of TNF-α induced expression of ICAM-1 by four 7-hydroxychromone-3-yl-acrylates **9a–d** is almost the same as the trend of inhibition by four chromone-3-yl-acrylates **8a–d**. Thus ethyl *trans*-3-(7-hydroxy-4-oxo-4*H*-1-benzopyran-3-yl)-acrylate (**9a**) and allyl *trans*-3-(7-hydroxy-4-oxo-4*H*-1-benzopyran-3-yl)-acrylate (**9c**) are the most active compounds among the four 7-hydroxychromone-3-yl-acrylates **9a–d**. From the structure–activity study, ethyl *trans*-3-(4-oxo-4*H*-1-benzopyran-3-yl)-acrylate (**8a**) was found to be the most potent in inhibiting the TNF-α induced expression of ICAM-1 on endothelial cells and was chosen for further study. Simple chromone was found to be least active in inhibiting the TNF-α induced expression of ICAM-1 on endothelial cells (Table 1).

2.4. Chromone derivative 8a inhibits TNF-α induced expression of VCAM-1 and E-selectin in addition to ICAM-1

The effect of compound **8a** was examined on TNF-α induced expression of other cell adhesion molecules like VCAM-1 and E-selectin in addition to ICAM-1. Human umbilical cord vein endothelial cells were incubated with or without compound **8a** at various concentrations for 2 h prior to induction with TNF-α (10 ng/ml) for 16 h for VCAM-1 and 4 h for E-selectin. As detected by cell-ELISA, CAMs were expressed at low levels on unstimulated endothelial cells and that were induced almost 3- to 4-fold upon stimulation with TNF-α. Interestingly, treatment of cells with compound **8a** led to a significant reduction in the TNF-α induced expression of ICAM-1, VCAM-1, and E-selectin (Fig. 1). Further, we found that compound **8a** inhibited these molecules in a concentration dependent manner. The inhibition of TNF-α induced expression of ICAM-1 was approximately 90%, whereas VCAM-1 and E-selectin were inhibited by more than 95% at 90 μg/ml (Fig. 2). The inhibition by compound **8a** remains unchanged if HUVECs were stimulated with LPS instead of TNF-α (data not shown).

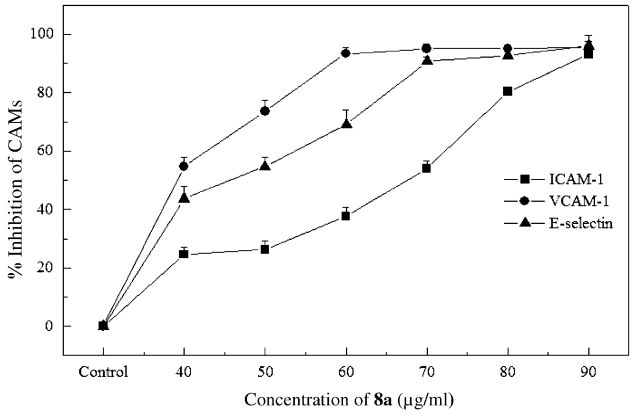


Figure 1. Inhibition of TNF-α induced ICAM-1, VCAM-1, and E-selectin expression by Compound **8a**: endothelial cells were grown to confluence in 96-well plates and incubated with or without indicated concentrations of compound **8a** for 2 h prior to induction with TNF-α (10 ng/ml) for 16 h for ICAM-1, VCAM-1 and 4 h for E-selectin. The ICAM-1, VCAM-1, and E-selectin levels on the cells were measured by ELISA as described in Section 4. The data presented are representative of three independent experiments. Values shown are means ± SD of quadruplicate wells.

The inhibitory activity of compound **8a** on ICAM-1, VCAM-1, and E-selectin expression was further confirmed by flow cytometry (Fig. 2a–c). As detected by ELISA and confirmed by flow cytometry, the unstimulated cells expressed low levels of ICAM-1, VCAM-1, and E-selectin. Upon stimulation with TNF-α, a substantial increase (3- to 4-fold) in the expression of all these three molecules was observed

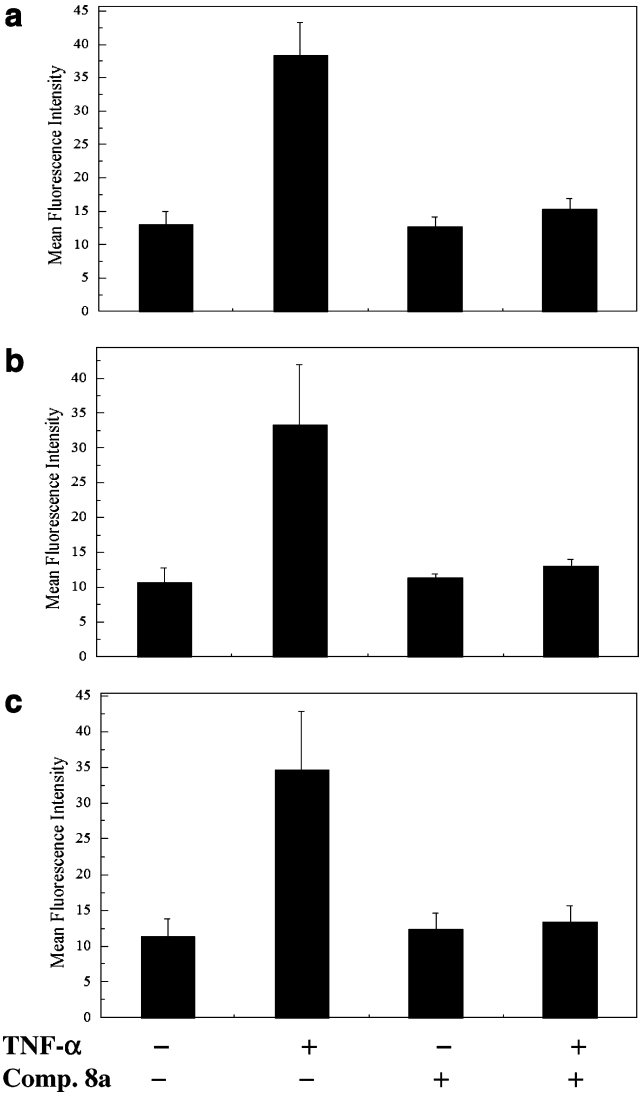


Figure 2. Flow cytometry analysis of inhibition of TNF-α induced ICAM-1, VCAM-1, and E-selectin expression by Compound **8a**: the endothelial cells were treated with 90 μg/ml of compound **8a** for 2 h, followed by stimulation with TNF-α (10 ng/ml) for 16 h for VCAM-1 and ICAM-1 and for 4 h for E-selectin. Expression of these molecules was measured by flow cytometry as described in Section 4. The data presented as means ± SD of three independent experiments after autofluorescence was subtracted from treated conditions. Cell Quest Software was used for statistical analysis ($p < 0.05$). Figure 2a–c represent ICAM-1, VCAM-1, and E-selectin inhibition, respectively.

(Fig. 2a–c). However, pre-treatment of endothelial cells with compound **8a** at 90 μg/ml concentration significantly inhibited TNF-α induced expression of ICAM-1, VCAM-1, and E-selectin (95%) (Fig. 2a–c).

2.5. Chromone derivative 8a inhibits TNF-α induced adhesion of neutrophils to endothelial monolayer

Neutrophil adhesion to endothelial monolayer requires a series of interactions; these interactions are mediated by cell adhesion molecules. To check the cell adhesion molecule's inhibitory activity with its functional consequence, neutrophil adhesion assay was performed as mentioned in Section 4. As detected by colorimetric assay, there was low adherence of neutrophils on unstimulated endothelial cells. This adherence was induced more than 3-fold by stimulation with TNF-α (data not shown). Interestingly, compound **8a** significantly inhibited the adhesion of neutrophils to endothelium in a concentration dependent manner and maximum 65% inhibition was calculated at concentration of 90 μg/ml (Fig. 3).

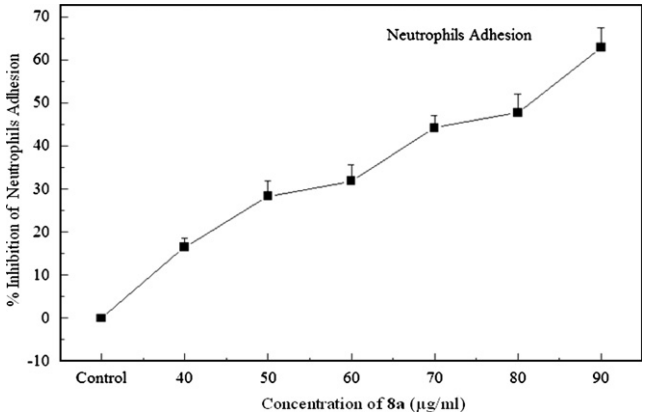


Figure 3. Inhibition of neutrophil adhesion to endothelium by compound **8a**: endothelial cells were grown to confluence in 96-well plates and incubated with or without indicated concentrations of compound **8a** for 2 h prior to induction with TNF-α (10 ng/ml) for 6 h. The adhesion of neutrophils on the cells was measured by colorimetric assay as described in Section 4. The data presented are representative of three independent experiments. Values shown are means ± SD of three independent experiments.

2.6. Chromone derivative 8a blocks TNF-α induced nuclear translocation of p65

Inflammatory cytokine TNF-α has been shown to cause rapid phosphorylation and degradation of IκBα, a cytoplasmic inhibitor of NF-κB, resulting in translocation of the activated p50/p65 heterodimer from the cytoplasm to the nucleus. To elucidate the mechanism of cell adhesion molecules inhibition by compound **8a**, we measured the levels of p65 in the cytoplasmic and the nuclear extracts prepared from compound **8a** treated cells using Western blot as mentioned in Section 4. It was observed that low levels of p65 were present in the nucleus of the unstimulated cells or cells treated with compound **8a** alone (Fig. 4, lanes 5 and 7), while comparatively higher levels were observed in the cytoplasm (Fig. 4, lanes 1 and 3). Upon stimulation with TNF-α the levels of p65 in the cytoplasm were decreased (Fig. 4, lane 2), while its levels were increased in the nucleus (Fig. 4, lane 6). On the other hand, upon treatment of the cells with compound **8a** prior to induction with TNF-α, the levels of p65 did not decrease significantly in the cytoplasm (Fig. 4, lane 4) and there was no concomitant increase in the p65 levels in the nucleus (Fig. 4, lane 8). These results therefore indicate that compound **8a** blocks the translocation of p65 from cytoplasm to the nucleus, and hence may be responsible for preventing the induction of ICAM-1, VCAM-1, and E-selectin by TNF-α.

2.7. Chromone derivative 8a attenuates TNF-α induced NF-κB activation

To examine further whether compound **8a** inhibits NF-κB activation, we performed electrophoretic mobility shift assays as mentioned in Section 4. Nuclear extracts were prepared from compound **8a** treated endothelial cells and assayed for NF-κB activation. As shown in Figure 5, upon stimulation with TNF-α the intensity of the NF-κB-shifted band was increased (lane 2 vs lane 3). Although compound **8a** alone had no effect on the basal level of NF-κB (lane 5), however, the treatment of cells with compound **8a** prior to induction with TNF-α caused a substantial decrease in the intensity of the shifted band (lane 4). The specificity of the NF-κB–DNA complex was confirmed in control experiments, where nuclear extracts were incubated with excess unlabeled oligos. Unlabeled NF-κB oligos inhibited the formation of the complex (lane 7), whereas

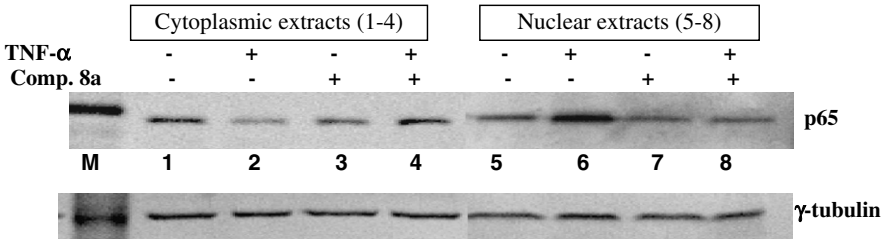


Figure 4. Compound **8a** blocks p65 translocation: the endothelial cells were incubated with or without 90 μg/ml concentration of compound **8a** for 2 h followed by induction with TNF-α (10 ng/ml) for 30 min. The cytoplasmic and nuclear extracts were prepared. Western blots for p65 were performed as described in Section 4. Lanes 1 and 5, unstimulated cells; lanes 2 and 6, stimulated with TNF-α; lanes 3 and 7, compound **8a** alone; lanes 4 and 8, stimulated with TNF-α after compound **8a** pre-treatment for 2 h. The data presented are representative of three independent experiments. γ-tubulin is used as loading control.

competition with an excess of an irrelevant oligonucleotide, Oct-1 or SP-1, did not inhibit the complex (compare lanes 6 and 8 with lane 7). These results indicate that compound **8a** significantly inhibits TNF- α induced NF-κB translocation and activation, and thus blocks the expression of cell adhesion molecules.

2.8. Chromone derivatives inhibit NADPH-catalyzed microsomal 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. The effects of chromone and its various analogs, **8a–d** and **9a–d**, on NADPH-catalyzed rat liver microsomal lipid peroxidation were examined as described in Section 4. We observed that chromone analogs inhibit lipid peroxidation in variable amounts (Table 2). Interestingly, compound **8a** was found to be most effective in inhibiting NADPH-catalyzed lipid peroxidation quite similar to ICAM-1 inhibition (Tables 1 and 2).

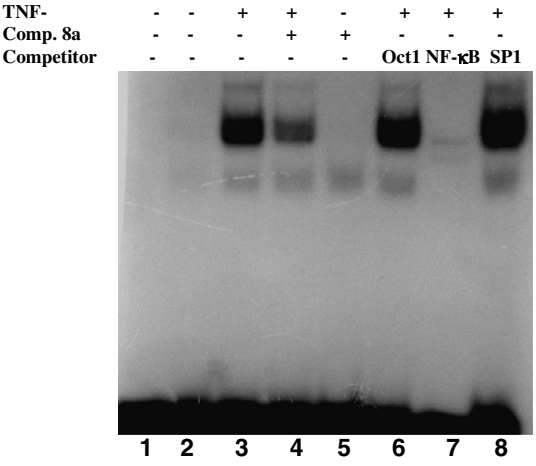


Figure 5. Inhibition of NF-κB activation by compound **8a**: the endothelial cells were incubated with or without 90 μg/ml concentration of compound **8a** for 2 h, followed by induction with TNF-α (10 ng/ml) for 30 min. The nuclear extracts were prepared. EMSA was performed as described in Section 4. Lane 1, free probe; lane 2, unstimulated cells; lane 3, stimulated with TNF-α; lane 4, stimulated with TNF-α after compound **8a** pre-treatment for 2 h; lane 5, compound **8a** alone; lane 6, cold chase with non-specific oligos Oct1; lane 7, cold chase with specific oligos NF-κB; lane 8, cold chase with non-specific oligos SP1. The data presented are representative of three independent experiments.

3. Discussion

Activation of vascular endothelium results in the release of various vascular cytokines such as interleukin 1β (IL-1β), tumor necrosis factor α (TNF-α), etc. These cytokines in turn induce the cell surface expression of cell adhesion molecules, which are centrally involved in endothelial recruitment of leukocytes.[42] Thus cell adhesion molecules play a major role in pathophysiology of inflammatory diseases. Our group previously reported that many natural and synthetic molecules were able to abrogate the induced expression of cell adhesion molecules. These findings provide a significant molecular basis for mode of action of biologically active compounds present in diet in preventing inflammation.[11–15]

In the present study, we have reported the design, synthesis, and biological activity evaluation of eight chromone derivatives, having alkoxycarbonyl vinyl moiety at the C-3 carbon in order to compare the effect of alcohol moiety of acrylate ester and the presence or absence of hydroxyl group on the benzenoid ring of the heterocyclic core. All these chromone derivatives have been evaluated for their ability to inhibit the TNF-α induced expression of cell adhesion molecules using HUVECs. We have shown that these chromone derivatives inhibited the TNF-α induced expression of ICAM-1 (Table 1). Out of eight compounds tested for this activity, compound **8a** was found to be most capable in inhibiting the TNF-α induced expression of ICAM-1 with lowest IC_{50} (45 μg/ml). The comparison of ICAM-1 inhibitory activities of these derivatives indicates that the chain length of the alcohol moiety has a significant effect on the ICAM-1 inhibition. There is a decrease in activity with increase in chain length. These results are in agreement with various cinnamic acid ester analogs, where we observed that ethyl is the optimum length of the side chain for maximum activity.[14] Although our results, in general, are in agreement with those of Lotito and Frei,[43] there are some differences in experimental conditions as well as in observations. The ICAM-1 inhibitory and antioxidant activities obtained by us followed almost similar

Table 2. Effect of *trans*-3-(4-oxo-4*H*-1-benzopyran-3-yl)-acrylates **8a–d** and **9a–d** and chromone on NADPH-catalyzed rat liver microsomal lipid peroxidation initiation

Compound	% Inhibition of NADPH-catalyzed lipid peroxidation	Concentrations	
		μg/ml[a]	μM
Ethyl *trans*-3-(4-oxo-4*H*-1-benzopyran-3-yl)-acrylate (**8a**)	52.86	90	368
n-Propyl *trans*-3-(4-oxo-4*H*-1-benzopyran-3-yl)-acrylate (**8b**)	42.55	80	330
Allyl *trans*-3-(4-oxo-4*H*-1-benzopyran-3-yl)-acrylate (**8c**)	43.44	90	348
iso-Propyl *trans*-3-(4-oxo-4*H*-1-benzopyran-3-yl)-acrylate (**8d**)	50.81	70	271
Ethyl *trans*-3-(7-hydroxy-4-oxo-4*H*-1-benzopyran-3-yl)-acrylate (**9a**)	32.28	90	346
n-Propyl *trans*-3-(7-hydroxy-4-oxo-4*H*-1-benzopyran-3-yl)-acrylate (**9b**)	39.83	110	426
Allyl *trans*-3-(7-hydroxy-4-oxo-4*H*-1-benzopyran-3-yl)-acrylate (**9c**)	13.27	100	364
iso-Propyl *trans*-3-(7-hydroxy-4-oxo-4*H*-1-benzopyran-3-yl)-acrylate (**9d**)	26.22	60	218
Chromone	25.40	90	616

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

pattern, though ICAM-1 inhibition by these derivatives is much higher than their antioxidant activity.

We have also observed that the compound **8a** significantly inhibited the TNF-α induced expression of VCAM-1 and E-selectin, in addition to that of ICAM-1 as analyzed by cell-ELISA and confirmed by flow cytometry experiments (Figs. 1 and 2). This inhibition was effected in a concentration dependent manner. Further, we have observed that compound **8a** significantly (approximately 65%) inhibited the TNF-α induced adhesion of neutrophils to endothelium (Fig. 3), whereas the inhibition of ICAM-1, VCAM-1, and E-selectin was estimated to be in the range 90–95% (Figs. 1 and 2). This difference may be due to the fact that these three cell adhesion molecules are not the only key players in the interactions between leukocytes and endothelium. Some other molecules like P-selectin, L-selectin or MadCAM may play important roles in binding of neutrophils to endothelial monolayer.

The cytokine-induced activation of cell adhesion molecules takes place at the level of transcription through the action of nuclear transcription factor-κB (NF-κB), which is present in the cytoplasm with its inhibitory protein IκBα. Upon stimulation with various pro-inflammatory stimuli like TNF-α, IL-1β, LPS, and ROS, etc., IκBα gets phosphorylated and degraded. Activated NF-κB then translocates to the nucleus where it binds to the promoter of various genes including cell adhesion molecules.[44] Our results demonstrated that compound **8a** blocked the translocation and activation of NF-κB visualized Western blots and EMSA (Figs. 4 and 5). The well-known NF-κB and cell adhesion molecule inhibitors work at wide range of concentrations. For example, aspirin, mesalamine, and phenyl methimazole inhibit the TNF-α induced expression of VCAM-1 at IC_{50} 6000 μM, 16,000 μM, and 500 μM, respectively.[45–47] Similarly, diclofenac, *N*-acetylcysteine, and pyrrolidone dithiocarbamate are most effective at concentrations of 750 μM, 100 μM, and 1000 μM, respectively.[48,49] In comparison, the IC_{50} value of compound **8a** is around 165 μM. The concentration at which compound **8a** works is comparatively lower if one considers the above examples of lead molecules or drugs that are in clinical use. Therefore, our results indicate that the chromone **8a** is potentially effective and therefore, could be useful for further pharmaceutical studies. Furthermore, it is possible that compound **8a** could be effective in blocking the induction of protein kinase C and protein tyrosine kinase or a cyclic AMP-independent protein kinase A associated with activation of NF-κB. In any event, further upstream mechanism of action responsible for inhibition of cell adhesion molecules remains to be elucidated in the future.

In conclusion, we have demonstrated for the first time the design, synthesis, and biological activity of novel chromone derivatives. The most potent compound **8a** inhibits TNF-α induced expression of ICAM-1, VCAM-1, and E-selectin in a dose and time dependent manner. As a functional consequence of this, it also inhibits the adherence of neutrophils to endothelial monolayer. We have also demonstrated that this inhibition could be at least partly due to the blocking of NF-κB activation. Our results may stimulate further research on using this molecule as a template for developing lead molecule towards the development of better anti-inflammatory agents.

4. Experimental

4.1. Materials

Anti-ICAM-1, anti-E-selectin, anti-VCAM-1, and TNF-α were purchased from Pharmingen, USA. M-199 media, L-glutamine, endothelial cell growth factor, trypsin, Puck's saline, HEPES, *o*-phenylenediamine, and anti-mouse IgG-HRP were purchased from Sigma Chemical Co., USA. Fetal calf serum was purchased from Biological Industries, Israel. The reactions were monitored by TLC on precoated Merck silica gel $60F_{254}$ plates; the spots were detected either by UV light or by spraying with 5% alcoholic $FeCl_3$ solution. Silica gel (100–200 mesh) was used for column chromatography. Melting points were recorded in open capillaries in a sulfuric acid bath and are uncorrected. Fourier-transform infrared spectra (FT-IR) were recorded on a Perkin-Elmer model 2000 FT-IR spectrometer. KBr pellets were used for FT-IR study. UV–vis absorption spectra were recorded on a Perkin-Elmer spectrophotometer. ^1H and ^{13}C NMR spectra were recorded on Bruker AC-300 Avance spectrometer using TMS as internal standard. The chemical shift values are on δ scale and the coupling constant values (J) are in Hz. The mass spectra were recorded on JEOL JMS-AX 505 W high resolution mass spectrometer. HRMS were done on a micro OTOF-Q instrument (Bruker Daltonics, Bremen) using electrospray in positive mode and JEOL AX505 instrument using electron impact (EI) ionization.

4.2. General procedure for synthesis of trans-3-(4-oxo-4H-1-benzopyran-3-yl)acrylates 8a–d and 9a–d

A mixture of **6** (1 g, 5.0 mmol)/**7** (1 g, 4.3 mmol), appropriate alcohol (50 ml), and concd sulfuric acid (2–3 drops) was refluxed at 100 °C. After the reaction was over as indicated by TLC, the reaction mixture was allowed to cool to room temperature and then poured into crushed ice and stirred vigorously, and the precipitated solid was filtered which afforded the desired esters **8a–d/9a–d**. The solid was washed with water and then recrystallized from ethanol.

4.3. Ethyl trans-3-(4-oxo-4H-1-benzopyran-3-yl)-acrylate (8a)

It was obtained as a yellow solid after recrystallization from ethanol in 98% yield, mp: 96–98 °C; UV (MeOH) λ_{max}: 290 and 267 nm; IR (KBr): 1706 (CO), 1666 (OCO), 1562, 1465, 1356, 1299, 1266, 1159, 1033, 988, 759, and 688 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz): δ 1.33 (t, 3H, J = 7.1 Hz, OCH_2CH_3), 4.25 (q, 2H, J = 7.1 Hz, OCH_2CH_3), 7.24 (m, 1H, C—8H), 7.28 (d, 1H, J = 15.8 Hz, C—2′H), 7.42 (d, 1H, J = 15.8 Hz, C—1′H),

7.44–7.72 (m, 2H, C—6H and C—7H), 8.11 (s, 1H, C—2H) and 8.27 (d, 1H, J = 7.8 Hz, C—5H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 14.5 (OCH_2CH_3), 60.7 (OCH_2CH_3), 118.3 (C-2′), 119.7 (C-10), 122.5 (C-8), 124.5 (C-3), 126.08 (C-6 and C-7), 126.64 (C-5), 135.5 (C-1′), 155.8 (C-9), 157.45 (C-2), 167.59 (C-3′) and 176.13 (C-4); EIMS, *m/z* (% rel int): 244 [M^+] (90), 215 [$M-C_2H_5$]$^+$ (88), 199 [$M-OC_2H_5$]$^+$ (100), 173 (30), 171 (97), 142 (18), 121 (85), 115 (98), 92 (98), 79 (98) and 62 (97). HRMS [M+Na]$^+$ calcd for $C_{14}H_{12}O_4$: 267.0633, found: 267.0622.

4.4. n-Propyl trans-3-(4-oxo-4H-1-benzopyran-3-yl)-acrylate (8b)

It was obtained as a white solid after recrystallization from ethanol in 98% yield, mp: 104–106 °C; UV (MeOH) λ_{max}: 263 and 289 nm; IR (KBr): 1708 (CO), 1660 (OCO), 1562, 1465, 1414, 1356, 1292, 1267, 1159, 1001, 858 and 764 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 1.0 (t, 3H, J = 7.2 Hz, $OCH_2CH_2CH_3$), 1.71 (m, 2H, OCH_2CH_2 CH_3), 4.16 (t, 2H, J = 7.31 Hz, $OCH_2CH_2CH_3$), 7.29 (d, 1H, J = 16.2 Hz, C—2′H), 7.42 (d, 1H, J = 16.2 Hz, C—1′H), 7.45 (br m, 2H, aromatic), 7.69 (s, 1H, C—2H), 8.12 (m, 1H, C—7H) and 8.27 (d, 1H, J = 7.9 Hz, C—5H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 10.32 ($OCH_2CH_2CH_3$), 21.95 ($OCH_2CH_2CH_3$), 66.03 ($OCH_2CH_2CH_3$), 117.98 (C-8), 119.29 (C-2′), 122.19 (C-3 and C-6), 125.71 (C-10), 126.24 (C-5), 133.87 (C-7), 135.15 (C-1′), 155.44 (C-2), 157.16 (C-9), 167.35 (C-3′) and 175.8 (C-4); EIMS, *m/z* (% rel int): 258 [$M]^+$ (46), 215 [$M-C_3H_7$]$^+$ (72), 199 [$M-OC_3H_7$]$^+$ (100), 171 (99), 142 (10), 121 (36), 115 (86), 92 (43), 79 (37) and 62 (30). HRMS [M+Na]$^+$ calcd for $C_{15}H_{14}O_4$: 281.0790, found: 281.0786.

4.5. Allyl trans-3-(4-oxo-4H-1-benzopyran-3-yl)-acrylate (8c)

It was obtained as a white solid after recrystallization from ethanol in 95% yield, mp: 118–120 °C; UV (MeOH) λ_{max}: 290 and 267 nm; IR (KBr): 1709 (CO), 1646 (OCO), 1560, 1465, 1412, 1359, 1290, 1242, 1153, 991, 859 and 760 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 4.70 (br s, 2H, $OCH_2CH{=}CH_2$), 5.24–5.39 (m, 2H, $OCH_2CH{=}CH_2$), 5.97 (br s, 1H, $OCH_2CH{=}CH_2$), 7.22 (m, 1H, C—8H), 7.28 (d, 1H, J = 15.6 Hz, C—2′H), 7.46 (d, 1H, J = 15.6 Hz, C—1′H), 7.53–8.23 (m, 3H, Ar—H) and 8.36 (s, 1H, C—2H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 64.8 ($OCH_2CH{=}CH_2$), 117.8 ($OCH_2CH{=}CH_2$), 118.14 (C-8), 118.83 (C-3), 122.28 (C-6), 123.93 (C-2′), 125.75 (C-5), 132.21 (C-7), 135.04 ($OCH_2CH{=}CH_2$), 136.04 (C-1′), 155.36 (C-2), 157.63 (C-9), 166.5 (C-3′) and 175.63 (C-4); EIMS, *m/z* (% rel int): 256 [$M]^+$ (12), 215 [$M-C_3H_5$]$^+$ (26), 199 [$M-OC_3H_5$]$^+$ (35), 171 (100), 142 (10), 121 (26), 115 (50), 92 (44), 77 (17) and 62 (30). HRMS [M+Na]$^+$ calcd for $C_{15}H_{12}O_4$: 279.3251, found: 279.3303.

4.6. Isopropyl trans-3-(4-oxo-4H-1-benzopyran-3-yl)-acrylate (8d)

It was obtained as a white solid after recrystallization from ethanol in 95% yield, mp: 130–135 °C; UV (MeOH) λ_{max}: 290 and 267 nm; IR (KBr): 1709 (CO), 1646 (OCO), 1615, 1560, 1465, 1412, 1359, 1290, 1242, 1153, 991, 859 and 760 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 1.24 [d, 6H, J = 5.56 Hz, $OCH(CH_3)_2$], 4.98 [m, 1H, $OCH(CH_3)_2$], 7.13 (d, 1H, J = 15.7 Hz, C—2′H), 7.44 (d, 1H, J = 15.8 Hz, C—1′H), 7.52–8.12 (m, 4H, Ar—H) and 8.86 (s, 1H, C—2H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 22.0 [$OCH(CH_3)_2$], 67.64 [$OCH(CH_3)_2$], 118.34 (C-8), 118.84 (C-3), 120.99 (C-2′), 125.7 (C-6), 126.4 (C-10), 134.9 (C-5), 136.17 (C-7), 136.36 (C-1′), 155.42 (C-2), 160.32 (C-9), 166.26 (C-3′) and 175.62 (C-4); EIMS, *m/z* (% rel int): 258 [$M]^+$ (12), 215 [$M-C_3H_7$]$^+$ (63), 199 [$M-OC_3H_7$]$^+$ (89), 171 (100), 142 (10), 121 (52), 115 (94), 92 (76), 79 (37) and 62 (55). HRMS [M+Na]$^+$ calcd for $C_{15}H_{14}O_4$: 281.0790, found: 281.0776.

4.7. Ethyl trans-3-(7-hydroxy-4-oxo-4H-1-benzopyran-3-yl)-acrylate (9a)

It was obtained as a white solid after recrystallization from ethanol in 92% yield, mp: 240–242 °C; UV (MeOH) λ_{max}: 271 nm; IR (KBr): 3152 (OH), 1707 (CO), 1652 (OCO), 1596, 1455, 1418, 1289, 1172, 1099, 956, 843 and 784 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 1.30 (t, 3H, J = 7.08 Hz, OCH_2CH_3, 4.22 (m, 2H, OCH_2CH_3), 6.85 (s, 1H, C—8H), 6.93 (d, 1H, J = 8.4 Hz, C—6H), 7.2 (d, 1H, J = 15.8 Hz, C—2′H), 7.4 (d, 1H, J = 15.8 Hz, C—1′H), 8.02 (d, 1H, J = 8.5 Hz, C—5H) and 8.2 (s, 1H, C—2H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 14.2 (OCH_2CH_3), 60.8 (OCH_2CH_3), 102.6 (C-8), 114.0 (C-6 and C-10),115.7 (C-3), 118.5 (C-2′), 121.0 (C-5), 136.0 (C-1′), 157.25 (C-2), 157.44 (C-9), 163.13 (C-7), 167.02 (C-3′) and 175.11 (C-4); EIMS, *m/z* (% rel int): 260 [M^+] (12), 231 [$M-C_2H_5$]$^+$ (8), 215 [$M-OC_2H_5$]$^+$ (20), 187 (80), 171 (62), 149 (18), 129 (32), 111 (36), 97 (58), 83 (78) and 69 (100). HRMS [EIMS] calcd for $C_{14}H_{12}O_5$: 260.0685, found: 260.0682.

4.8. Propyl trans-3-(7-hydroxy-4-oxo-4H-1-benzopyran-3-yl)-acrylate (9b)

It was obtained as a white solid after recrystallization from ethanol in 92% yield, mp: 176–180 °C; UV (MeOH) λ_{max}: 272, 261 and 255 nm; IR (KBr): 3234 (OH), 1706 (CO), 1652 (OCO), 1596, 1451, 1417, 1284, 1164, 1099, 955, 846 and 784 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 1.0 (t, 3H, J = 7.08 Hz, $OCH_2CH_2CH_3$), 1.71 (m, 2H, $OCH_2CH_2CH_3$), 4.12 (t, 2H, J = 6.18 Hz, $OCH_2CH_2CH_3$), 6.84 (d, 1H, J = 8.65 Hz, C—8H), 6.93 (d, 1H, J = 8.64 Hz,C—6H), 7.22 (d, 1H, J = 15.9 Hz, C—2′H), 7.42 (d, 1H, J = 15.9 Hz, C—1′H), 8.01 (d, 1H, J = 7.8 Hz, C—5H) and 8.28 (s, 1H, C—2H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 10.4 ($OCH_2CH_2CH_3$), 21.9 ($OCH_2CH_2CH_3$), 65.7 ($OCH_2CH_2CH_3$), 102.6 (C-8), 115.7 (C-6), 118.4 (C-10), 120.9 (C-2′), 122.0 (C-3), 127.3 (C-5), 136.1 (C-1′), 155.1 (C-2), 157.7 (C-9), 163.2 (C-7), 167.1 (C-3′) and 176.0 (C-4); EIMS, *m/z* (% rel int): 274 [$M]^+$ (16), 231 [$M-C_3H_7$]$^+$ (18), 215 [$M-OC_3H_7$]$^+$ (48), 187 (100), 171 (10), 158 (6), 131 (17), 108 (7) and 79 (16). HRMS [EIMS] calcd for $C_{15}H_{14}O_5$: 274.0841, found: 274.0855.

4.9. Allyl trans-3-(7-hydroxy-4-oxo-4H-1-benzopyran-3-yl)-acrylate (9c)

It was obtained as a white solid after recrystallization from ethanol in 95% yield, mp: 232–234 °C; UV (MeOH) λ_{max}: 290 and 267 nm; IR (KBr): 3185 (OH), 1712 (CO), 1654 (OCO), 1453, 1287, 1157, 1100, 989, 835 and 782 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz): δ 4.65 (br s, 2H, $OCH_2CH{=}CH_2$), 5.22–5.37 (m, 2H, $OCH_2CH{=}CH_2$), 5.96 (br s, 1H, $OCH_2CH{=}CH_2$), 6.86 (s, 1H, C—8H), 6.92 (d, 1H, J = 7.8 Hz, C—6H), 7.21(d, 1H, J = 15.8 Hz, C—2′H), 7.46 (d, 1H, J = 16.0 Hz, C—1′H), 7.96 (d, 1H, J = 7.6 Hz, C—5H) and 8.67 (s, 1H, C—2H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 64.71 ($OCH_2CH{=}CH_2$), 102.7 ($OCH_2CH{=}CH_2$ and C-8), 115.9 (C-6 and C-10), 117.9 (C-3), 120.0 (C-2′), 127.48 (C-5), 132.9 ($OCH_2CH{=}CH_2$), 137.2 (C-1′), 157.2 (C-9), 159.5 (C-2), 162.9 (C-7), 163.37 (C-3′) and 175.3 (C-4); EIMS, m/z (% rel int): 272 $[M]^+$ (10), 231 $[M-C_3H_5]^+$ (10), 215 $[M-OC_3H_5]^+$ (20), 207 (14), 187 (100), 171 (30), 167 (36), 137 (12), 97 (28), 84 (40) and 70 (46). HRMS [EIMS] calcd for $C_{15}H_{12}O_5$: 272.0685, found: 272.0697.

4.10. Isopropyl trans-3-(7-hydroxy-4-oxo-4H-1-benzopyran-3-yl)-acrylate (9d)

It was obtained as a white solid after recrystallization from ethanol in 95% yield, mp: 250–252 °C; UV (MeOH) λ_{max}: 271 nm; IR (KBr): 3187 (OH), 1702 (CO), 1654 (OCO), 1594, 1451, 1354, 1289, 1231, 1170, 1099, 998, 845, 783 and 687 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz): δ 1.27 [d, 6H, J = 4.8 Hz, $OCH(CH_3)_2$], 5.01 [m,1H, $OCH(CH_3)_2$], 6.87 (s, 1H, C—8H), 6.95 (d, 1H, J = 7.6 Hz, C—6H), 7.14 (d,1H, J = 15.7 Hz, C—2′H), 7.43 (d, 1H, J = 15.7 Hz, C—1′H), 7.97 (d, 1H, J = 7.6 Hz, C—5H) and 8.7 (s, 1H, C—2H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 22.04 [$OCH(CH_3)_2$], 60.26 [$OCH(CH_3)_2$], 102.74 (C-8), 115.9 (C-6 and C-10), 117.5 (C-3), 118.1 (C-2′), 120.7 (C-5), 136.5 (C-1′), 159.4 (C-2 and C-9), 163.3 (C-7), 166.3 (C-3′) and 175.3 (C-4); EIMS, m/z (% rel int): 274 $[M]^+$ (12), 231 $[M-C_3H_7]^+$ (13), 215 $[M-OC_3H_7]^+$ (20), 187 (100), 158 (10), 137 (8), 131 (10), 108 (10) and 79 (16). HRMS $[M+Na]^+$ calcd for $C_{15}H_{14}O_5$: 297.0739, found: 297.0714.

4.11. Cells and cell culture

Primary endothelial cells were isolated from human umbilical cord using mild trypsinization.[17] The cells were grown in M-199 medium supplemented with 15% heat inactivated fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin B, and endothelial cell growth factor (50 μg/ml). At confluence, the cells were subcultured using 0.05% trypsin-0.01 M EDTA solution and were used between passages three to four.

4.12. Cell viability assay

The cytotoxicity of chromone derivatives was analyzed by using trypan blue exclusion test and it was further confirmed by colorimetric MTT (methylthiazolydiphenyl-tetrazolium bromide) assay as described.[14] Briefly, endothelial cells were treated with DMSO alone (0.25%, as vehicle) or with different concentrations of chromone derivatives for 24 h. Four hours before the end of incubation, medium was removed and 100 μl MTT (5 mg/ml in serum free medium) was added to each well. The MTT was removed after 4 h, cells were washed out with PBS, and 100 μl DMSO was added to each well to dissolve water insoluble MTT-formazan crystals. Absorbance was recorded at 570 nm in an ELISA reader (Bio-Rad, Model 680, USA). All experiments were performed at least 3 times in triplicate wells. DMSO was used as solvent (vehicle) for dissolving all the compounds tested. DMSO concentration (0.25%) was held constant in all the experiments and this small concentration did not cause any cytotoxicity on endothelial cells (Table 1).

4.13. Modified ELISA for measurement of ICAM-1, VCAM-1, and E-selectin

Cell-ELISA was used for measuring the expression of ICAM-1, VCAM-1, and E-selectin on surface of endothelial cells.[14] Endothelial cells were incubated with or without the test compounds at desired concentrations for the required period, followed by treatment with TNF-α (10 ng/ml) for 16 h for ICAM-1 and VCAM-1 expression and for 4 h for E-selectin. The cells were fixed with 1.0% glutaraldehyde. Non-specific binding of antibody was blocked by using skimmed milk (3.0% in PBS). Cells were incubated overnight at 4 °C with anti-ICAM-1, anti-VCAM-1, and anti-E-selectin mAbs, diluted in blocking buffer, the cells were further washed with PBS and incubated with peroxidase-conjugated goat anti-mouse secondary Abs. After washings cells were exposed to the peroxidase substrate (*o*-phenylenediamine dihydrochloride 40 mg/100 ml in citrate phosphate buffer, pH 4.5). Reaction was stopped by the addition of 2 N sulfuric acid and absorbance at 490 nm was measured using microplate reader (Spectramax 190, Molecular Devices, USA). The optical density (A490) for each control at each concentration of compound was set to unity and then the change in relative–fold induction was calculated. The percentage inhibition was calculated as [1-A490 (increasing compounds) divided by the A490 (control)] × 100. The data presented are in the form of curve graphs, plotted between concentrations vs. percentage inhibition.

4.14. Flow cytometry analysis

The cell surface expression of ICAM-1, VCAM-1, and E-selectin on endothelial cells was analyzed by flow cytometry.[14] The endothelial cells were incubated with or without compound **8a** for 2 h. The cells were further treated with TNF-α (10 ng/ml) and incubated for 16 h for ICAM-1 and VCAM-1, and for 4 h for E-selectin expression. The cells were washed with PBS and dislodged, following which they were incubated with anti-ICAM-1, anti-VCAM-1, and anti-E-selectin (1.0 μg/10^6 cells, 30 min, 4 °C). After incubation, the cells were washed with PBS and then stained with FITC conjugated

goat anti-mouse IgG for 30 min at 4 °C. The cells were then fixed with 1.0% paraformaldehyde and were analyzed for estimating the expression of cell adhesion molecules using a flow cytometer (FACSVantage, Becton and Dickinson, USA). For each sample, 20,000 events were collected. Analysis was carried out by using Cell Quest Software (Becton–Dickinson, USA). The autofluorescence intensity was subtracted from treated conditions and mean fluorescence intensity was estimated from three independent experiments and bar diagrams were plotted.

4.15. Neutrophil isolation

Neutrophils were isolated from peripheral blood of healthy individuals.[14] Blood was collected in heparin solution (20 U/ml) and erythrocytes were removed by sedimentation against 6% dextran solution. Plasma, rich in white blood cells, was layered over Ficoll-Hypaque solution, followed by centrifugation (300 g for 20 min, 20 °C). The top saline layer and the Ficoll-Hypaque layer were aspirated leaving neutrophils/RBC pellet. The residual red blood cells were removed by hypotonic lysis. Isolated cells were washed with PBS and resuspended in PBS containing 5 mM glucose, 1 mM $CaCl_2$, and 1 mM $MgCl_2$ at a final concentration of 6×10^5 cells/ml.

4.16. Cell adhesion assay

Neutrophil adhesion assay was performed under static conditions as described previously.[14] Endothelial cells plated in 96-well culture plates were incubated with or without compound **8a** at desired concentrations for 2 h, followed by induction with TNF-α (10 ng/ml) for 6 h. Endothelial monolayers were washed with PBS and neutrophils (6×10^4/well) were added over it and were allowed to adhere for 1 h at 37 °C. The non-adherent neutrophils were washed with PBS and neutrophils bound to endothelial cells were assayed by adding a substrate solution consisting of *o*-phenylenediamine dihydrochloride (40 mg/100 ml in citrate phosphate buffer, pH 4.5), 0.1% cetitrimethyl ammonium bromide, and 3-amino-1,2,4 triazole (1 mM). The absorbance was read at 490 nm using an automated microplate reader (Model 680, Bio-Rad, USA). The percentage inhibition of neutrophil adhesion was calculated in same pattern as mentioned in ELISA. The data are presented in the form of curve graphs, plotted between concentrations versus percentage inhibition.

4.17. Preparation of cytoplasmic and nuclear extracts

Endothelial cells (2×10^6) were incubated with or without 90 μg/ml concentration of compound **8a** for 2 h, followed by induction with TNF-α (10 ng/ml) for 30 min.[8] The cells were washed with PBS, dislodged using a cell scraper, and centrifuged at 300 g for 10 min. The cell pellet was resuspended in cell lysis buffer (10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM PMSF, 1 mM DTT, 0.5% Nonidet P40, 0.1 mM EGTA, 0.1 mM EDTA, and cocktail of proteinase inhibitors) and allowed to swell on ice for 5 min. Following centrifugation at 3300*g* for 15 min, the supernatant was collected as cytoplasmic extract and stored at −70 °C. The nuclear pellet was resuspended in nuclear extraction buffer (20 mM HEPES, 25% glycerol, 1.5 mM $MgCl_2$, 420 mM NaCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM PMSF, 1 mM DTT, and cocktail of proteinase inhibitors) and incubated for 30 min at 4 °C. The extracted nuclei were pelleted at 25,000 g (30 min at 4 °C) and the supernatant was collected as nuclear extract. The protein concentration in the extracts was estimated by bicinchoninic acid (BCA) method.

4.18. Western blots of p65 and γ-tubulin

Following the experimental treatments of HUVECs, cytoplasmic and nuclear extracts were prepared as mentioned above and protein estimation was done. These extracts were subjected to 8% SDS–PAGE and transferred to nitrocellulose membrane in 25 mM Tris, 192 mM glycine, and 20% methanol at 35 V for 16 h. After blocking in 3% skimmed milk, membrane was incubated with anti-p65 or anti-γ-tubulin antibody (1:250, Santa Cruz, Biotechnology) for 2 h. Immunoreactive proteins were detected by HRP-conjugated anti-rabbit secondary antibody (1:500) incubated for 2 h. After intensive washing, membrane was developed with DAB as a substrate in 10 mM Tris–HCl buffer at pH 7.6.

4.19. NF-κB activation assay

To determine NF-κB activation, electrophoretic mobility shift assay (EMSA) was performed with some modifications in a previously published procedure.[8] Briefly, nuclear extracts were prepared from compound **8a** treated endothelial cells, 10 μg of nuclear extract was incubated with 40–80 fmoles of ^{32}P-end labeled double-stranded NF-κB oligonucleotide (5′-AGTTGA GGGGACTTTCCCAGG-3′) in binding buffer [12 mM HEPES, 50 mM NaCl, 10 mM Tris–HCl, pH 7.5, 10% glycerol, 1 mM EDTA, 1 mM DTT, and 1.0 μg poly(dI–dC)] for 30 min at RT. The DNA–protein complexes were analyzed by electrophoresis on a 4% native polyacrylamide gel using Tris–glycine buffer at pH 8.5 and visualized by autoradiography.

4.20. 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 previous method.[13] Male rats of Wistar strain weighing around 200 g were used for the preparation of liver microsome.[13] The assay of the initiation of lipid peroxidation has been described previously.[13] 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 the test compounds (100 μM each in 0.2 ml DMSO) were added, followed by incubation at 37 °C for 10 min. NADPH (0.5 mM) was added to the reaction mixture 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 color of the thiobarbituric acid reactive substance (TBRS) formed was measured at 535 nm. The lipid peroxidation was found to be linear up to 15 min under the conditions described here.

4.21. Statistical analysis

Results are given as means ± SD. Independent two-tailed Student's *t* test was performed. Differences were considered statistically significant for $p < 0.05$. The statistical analysis was performed using Microcal Origin software (ver 3.0; Microcal Software Inc, Northampton, MA and Cell Quest Software, Becton–Dickinson, USA).

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