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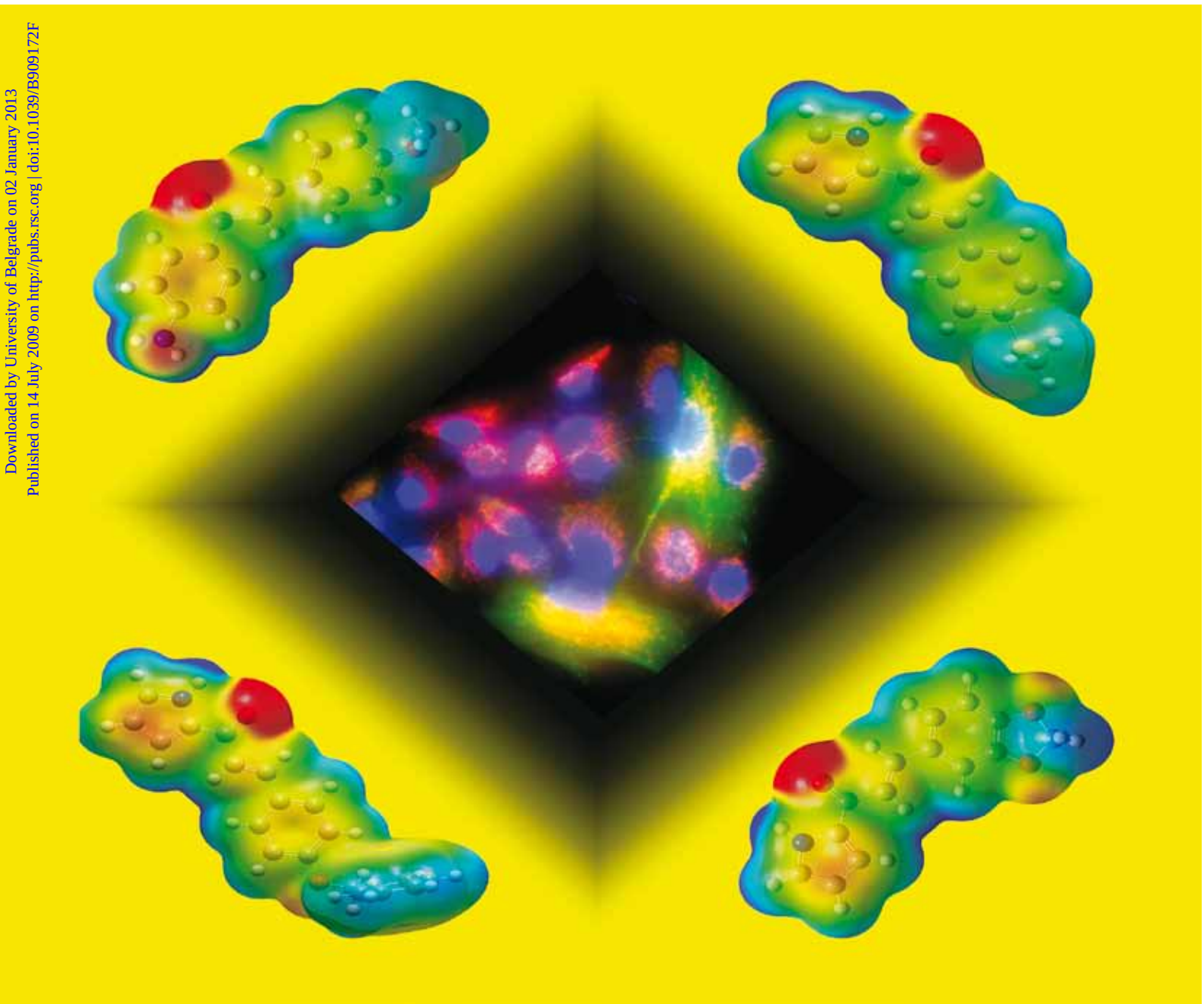

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PAPER

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Immunomodulatory effects of functionalised chalcones on pro-inflammatory cytokine release from lung epithelial cells

Immunomodulatory effects of functionalised chalcones on pro-inflammatory cytokine release from lung epithelial cells†

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Chalcones, prepared in high yield in polyethylene glycol, M_w = 300 (PEG300), were screened for inhibition of the thrombin-mediated release of pro-inflammatory interleukin-6 (IL-6) and interleukin-8 (IL-8) from the A549 lung epithelial cell line. Several show significant down-regulation of these pro-inflammatory mediators in a dose-dependant manner with one exhibiting pro-inflammatory properties. Computational studies on selected chalcones revealed a detailed understanding of the structure, electron density and biological activity relationship.

Introduction

Chalcone is a generic term given to organic compounds bearing the 1,3-diphenylprop-2-en-1-one framework. Natural and synthetic chalcones can have a wide range of biological activities which relate to the preferential reactivity of the α,β-unsaturated ketone moiety, as a soft electrophile, towards soft nucleophiles such as thiols. Chalcones are not susceptible to attack by strong nucleophiles, which include amino and hydroxyl groups in nucleic acids. This property circumvents problems associated with mutagenicity and carcinogenicity, in contrast to the mode of action of alkylating agents in cancer chemotherapy. The biological activity of chalcones encompasses cytotoxicity, anti-tumour, anti-plasmodial, antioxidant and anti-inflammatory properties.[1] Chronic airway diseases such as asthma and chronic obstructive pulmonary disease are characterised by marked inflammatory responses including pro-inflammatory cytokine release. An important tissue in this regard is the respiratory epithelium which releases IL-6 and IL-8 as well as other inflammatory mediators, all of which contribute to the pathology associated with chronic respiratory diseases. These mediators may be released from epithelial cells by a variety of stimuli including proteases such as thrombin, which often correlate with disease severity.[2] Hence, therapeutic strategies that target pro-inflammatory cytokine release and protease production from respiratory epithelium are of potential benefit in inflammatory lung diseases. Interestingly, many chalcone compounds exhibit anti-inflammatory properties through the inhibition of pro-inflammatory mediators such as interleukin-5 (IL-5), vascular cell adhesion molecule-1 (VCAM-1), prostaglandin E_2 (PGE_2) and nitric oxide (NO).[3] Herein, we evaluate a series of synthetic chalcone derivatives towards inhibition of thrombin-mediated release of IL-6 and IL-8 from the A549 respiratory epithelial cell line, establishing a structure, electron density and activity relationship. The choice of aromatic rings and substituents on the chalcone rings A and B relates to variation in electron densities, with variation on electronic properties of the α,β-unsaturated ketone moiety having a dramatic influence on biological activity. This is consistent with the computed electron density distributions in chalcones using lowest unoccupied molecular orbital (LUMO) and molecular electrostatic potential (MEP) maps, where the carbonyl carbon and β-carbon atoms are the most electron deficient.[4] Electron withdrawing groups on the A and B aromatic rings serve to enhance the electron deficiency of the β-carbon, and thus its reactivity towards nucleophiles. In addition, the reducibility of the carbonyl group relates to its electron density, with a readily reducible carbonyl group being electron deficient.

Results and discussion

Immunomodulatory effect of chalcones on thrombin-mediated IL-6 and IL-8 production from A549 lung epithelial cells

Base catalysed Claisen–Schmidt condensation in PEG300 gave access to chalcones in high yield (Table 1) while addressing green chemistry metrics. Compounds **1–5** and **9–12** (Table 1) were cytotoxic to the A549 lung epithelial cells between 200 and 300 μM, and accordingly the concentration range that was used for further investigation was 1–100 μM (see ESI†). Compound **8** was the only chalcone derivative to exhibit pro-inflammatory IL-6 and IL-8 release at both 50 μM and 100 μM (see ESI†). Compounds **6**, **7**, **9**, **10** and **12** showed significant down-regulation of thrombin-mediated IL-6 and IL-8 release at both 50 μM and 100 μM, with compound **7** exhibiting a significant down-regulation of thrombin-mediated IL-6 and IL-8 release at 10 μM (31% and 43%, respectively) (Fig. 1). Compounds **7** and **10** show the greatest down-regulation

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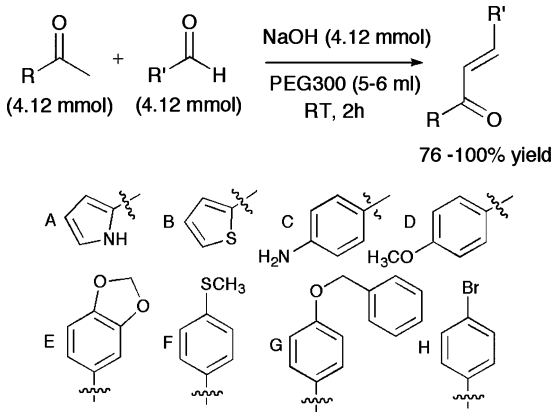
† Electronic supplementary information (ESI) available: Experimental procedures for the synthesis of chalcones, testing, computational analysis and additional figures. See DOI: 10.1039/b909172f

Table 1 Synthesis of chalcones



Compound	R	R′	Yield (%)
1	A	D	100
2	B	D	100
3	C	D	81
4	D	D	76
5	D	E	94
6	C	F	100
7	A	E	82
8	A	G	88
9	A	F	100
10	A	H	78
11	D	G	80
12	D	F	81

of IL-6 (88% and 100% reduction, respectively, at 50 μM; 100% reduction at 100 μM) and IL-8 (88% and 71% reduction, respectively, at 50 μM; 100% and 99% reduction, respectively, at 100 μM), with no significant discrimination between them. In contrast, compound **9** exhibited higher down-regulation of IL-6 than IL-8 (54% and 20% reduction, respectively, at 50 μM; 100% and 65% reduction, respectively, at 100 μM). Compound **6** showed down-regulation of IL-6 (45% reduction at 50 μM; 78% reduction at 100 μM) and IL-8 (37% reduction at 50 μM; 81% reduction at 100 μM). Compound **12** showed a 38% down-regulation of IL-8 at 50 μM and a 57% down-regulation at 100 μM. A non-specific protease assay (Azocoll) showed that the chalcone compounds did not inhibit the proteolytic activity of thrombin *per se* (see ESI†), indicating that these chalcones exhibited inhibitory effects further down the signalling pathway.

Computer-assisted conformational study of selected chalcones

Partially relaxed scan calculations reveal that the energies of rotamers depend essentially on the torsion angles φ and θ, with planar forms being highly preferred for the majority of compounds studied (see Table 2 and ESI†). Compounds **1** and **7–10** containing a pyrrol ring have a minimum energy conformation only when the nitrogen proton in the pyrrol ring is hydrogen bonded with the carbonyl oxygen. At RHF/6-31+G** level of theory, close to planar structures are maintained for compounds **1** and **7–10**, while a significant

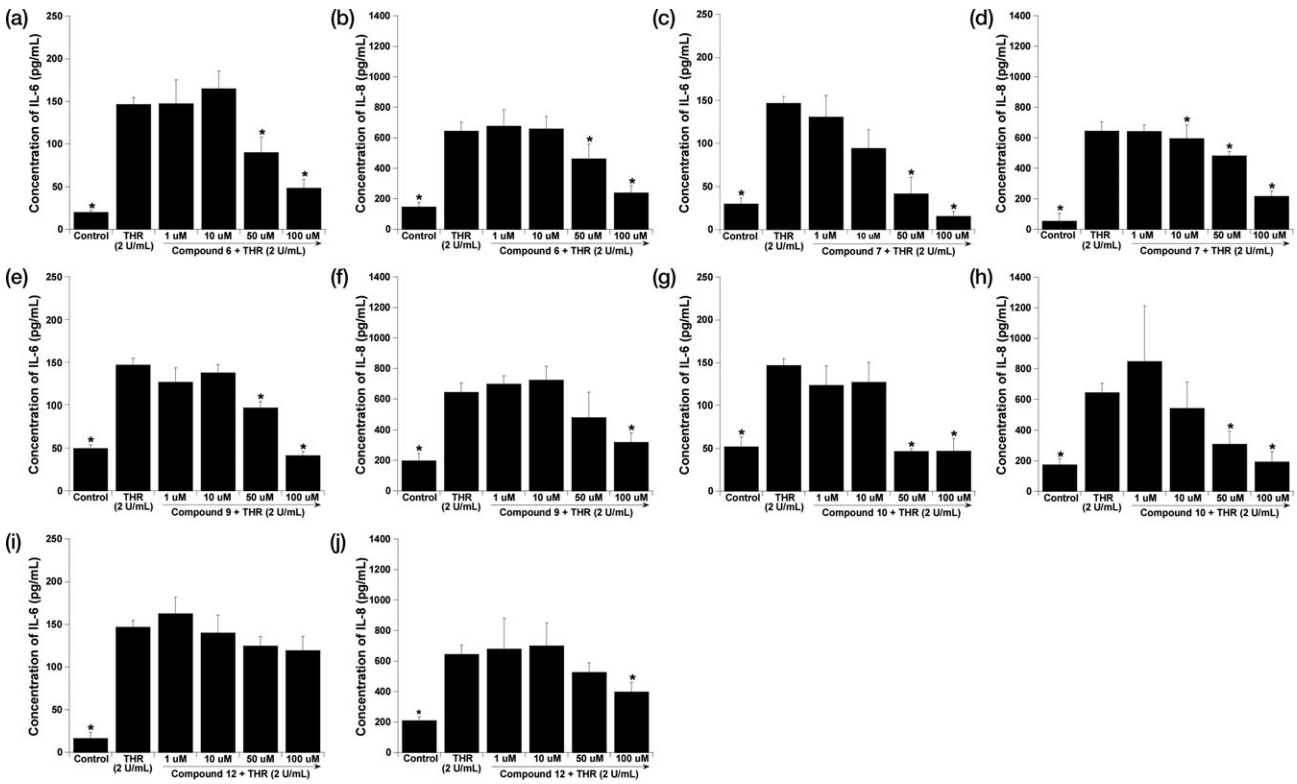
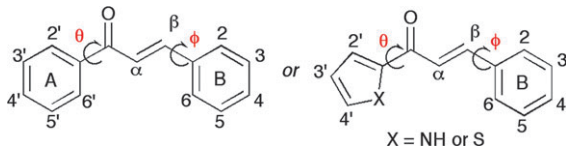


Fig. 1 The inhibition of thrombin-mediated interleukin-6 (a, c, e, g, i) and interleukin-8 (b, d, f, h, j) released from A549 respiratory epithelial cells pre-treated with compounds **6** (a, b), **7** (c, d), **9** (e, f), **10** (g, h) and **12** (i, j). Data presented are mean values ± SEM, $n = 3$. * significantly different from thrombin control (* $P < 0.008$).

Table 2 Torsion angles obtained for compounds **1**, **3**, **6–10** using different levels of theory



	RHF/3-21G torsion angles/°		RHF/6-31 + G** torsion angles/°	
Compound	ϕ	θ	ϕ	θ
1	−0.97	−0.03	3.57	0.07
3	−3.58	−0.81	−9.96	−14.51
6	−6.74	−1.00	−14.44	−14.10
7	−177.84	0.03	−178.49	0.04
8	−179.93	0.00	179.62	−0.02
9	−1.41	−0.03	−9.49	−0.20
10	−1.36	−0.02	−0.01	0.00

deviation from planarity is observed for compounds **3** and **6** which lack a pyrrol ring. The presence of a pyrrol ring strongly favours planar conformations, with the pyrrol nitrogen proton engaging in hydrogen bonding with the carbonyl oxygen. Previous studies by Lopez *et al.* demonstrated the importance of planarity as a conformational requirement of chalcones for their antifungal effects.[4] Indeed, planarity of the chalcones may be important in the current study.

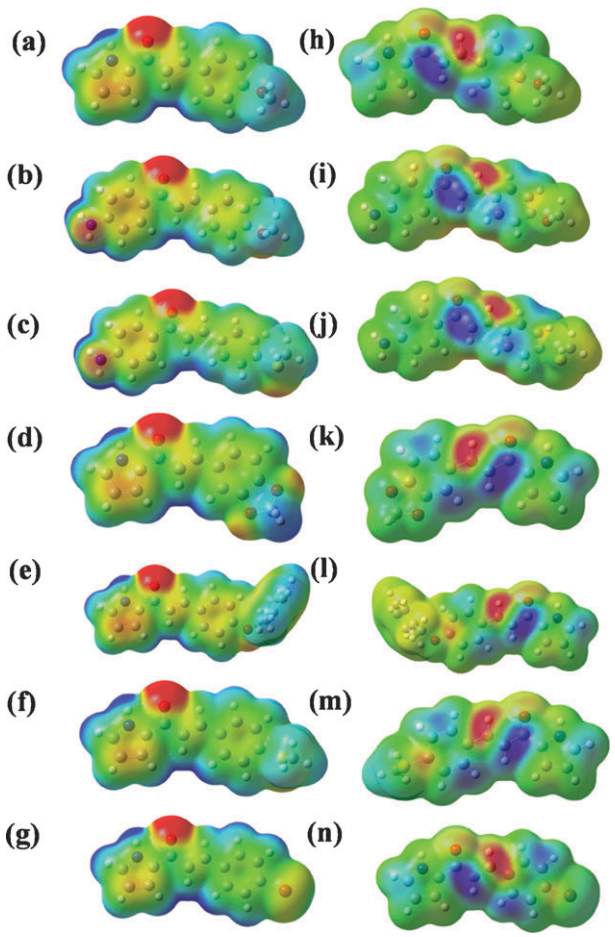


Fig. 2 MEP ((a)–(g)) and LUMO ((h)–(n)) maps for compounds **1** (a, h), **3** (b, i), **6** (c, j), **7** (d, k), **8** (e, l), **9** (f, m) and **10** (g, n) optimised at 6-31 + G**.

LUMO and MEP maps of selected chalcones

LUMO and MEP maps delineate electron deficient areas which could be critical to the possible mechanism of action (Fig. 2). The MEP isosurfaces of compounds **1**, **3** and **6–10** show that the most positive centres lie near to the β and carbonyl carbons (Fig. 2(a)–(g)), rendering the β and carbonyl carbons more susceptible to nucleophilic attack by enzymes or receptors. LUMO maps for compounds **1**, **3** and **6–10** show that regions with the highest absolute values of LUMO occur at the β and carbonyl carbons, which provide rich sites for nucleophilic attack (Fig. 2(h)–(n)).

Conclusions

From the data reported here, we found that chalcones possessing a planar conformation with electron withdrawing substituents on the B ring, compounds **7** and **10**, are most active in down-regulating thrombin-mediated release of IL-6 and IL-8 in A549 cells, with the β and carbonyl carbons being the most susceptible sites towards a possible nucleophilic attack by enzymes or receptors. Though the planarity of compound **8** is very similar to that of compounds **7** and **10**, it is thought that the presence of a bulky *para*-substituent, residing out of plane on ring B, results in a total loss of anti-inflammatory activity making compound **8** a pro-inflammatory compound instead. Compound **9** differs structurally from compounds **7** and **10** in the nature of ring B which is associated with a slight distortion from planarity in the torsion angle φ and decreased activity. In addition, this structural change dramatically reduces the ability of this compound to down-regulate IL-8, displaying a greater specificity of this compound for IL-6. Compound **6** differs from compound **9** in its ring A composition, where the pyrrol ring is replaced with a *para*-aminophenyl ring. The loss of hydrogen bonding from the pyrrol nitrogen proton with the carbonyl oxygen results in a significant deviation from

planarity as evident by the torsion angle θ at the RHF/6-31 + G** level of theory. The distortion from planarity in this compound is likely to be a contributor to its decreased activity compared to compounds **7**, **10** and **9**.

Chalcones are an important class of biologically active compounds, and the reported results represent a major advance in establishing a detailed understanding of a structure, electron density and biological activity relationship, showing a significant down-regulation of selected pro-inflammatory mediators in a dose-dependent manner. However, further studies will be required to determine the precise biological target involved in cytokine release within the cell.

Experimental

General experimental

4-Bromobenzaldehyde (99% purity), 4′-methoxyacetophenone (99% purity) and 2-acetylthiophene (≥ 98% purity) were purchased from Aldrich and used without further purification. Anisaldehyde (≥ 99.5% purity), piperonal (≥ 99% purity), 4-aminoacetophenone (99% purity) and PEG300 were purchased from Fluka and used without further purification. 4-(Methylthio)benzaldehyde (97% purity) and 2-acetylpyrrole (98% purity) were purchased from Alfa Aesar and used without further purification. ^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz) spectra were recorded on a Bruker AV500 instrument in 5 mm NMR tubes. Samples were recorded in DMSO-d^{6} solution in ppm (δ) and referenced to the internal residual partially-deuterated DMSO septet at 2.50 ppm (^{1}H NMR) and 39.52 ppm (^{13}C NMR). Mass spectrometry measurements were performed on a Waters Micromass Autospec Mass Spectrometer.

General route for the synthesis of chalcones

The chalcone compounds featured in the study were prepared using a base catalysed Claisen–Schmidt condensation reaction of an arylmethyl ketone and an aryl aldehyde using polyethylene glycol (PEG300) as a benign reaction medium. PEG's low toxicity, low volatility and biodegradability represent important environmentally benign characteristics, which are particularly attractive when combined with its relatively low cost as a bulk commodity chemical. The respective arylmethyl ketone (4.12 mmol) was added to a stirred solution of PEG300 (5–6 ml) and crushed sodium hydroxide (NaOH) (4.12 mmol) at ambient temperature. The respective aryl aldehyde (4.12 mmol) was then added and the reaction mixture was left stirring at room temperature for 2 h. Water (100 ml) was added, the precipitate was collected by suction filtration and dried on a high vacuum line.

Cytotoxicity studies of chalcones on A549 lung epithelial cells

The cytotoxicity of the chalcone derivatives on A549 lung epithelial cells was determined using acid phosphatase and lactate dehydrogenase assays. The A549 cells were seeded at 1.2×10^{5} per well in 24-well cell culture plates and allowed to grow to confluence in humidified atmosphere in air at 37 °C, 5% (v/v) CO_2. The culture medium was replaced with serum-free medium and incubated for 24 h. The cells were then exposed to the chalcone derivatives at 300 μM, 200 μM, 100 μM and 50 μM (DMSO < 1% [v/v]). After 24 h incubation at 37 °C, 5% CO_2, acid phosphatase (see ESI†) and lactate dehydrogenase assays (see ESI†) were performed.

Pro-inflammatory effect of chalcones on A549 lung epithelial cells

The chalcone derivatives were studied to determine if they had pro-inflammatory effects on A549 lung epithelial cells. Briefly, the A549 cells were seeded at 1.2×10^{5} per well in 24-well cell culture plates and allowed to grow to confluence in humidified atmosphere in air at 37 °C, 5% (v/v) CO_2. The culture medium was replaced with serum-free medium and cells incubated for 24 h. The cells were incubated in the presence of 100 μM, 50 μM, 10 μM and 1 μM of the chalcone derivatives for 24 h. The cell supernatants were collected and assayed for IL-6 and IL-8 using ELISA (see ESI†).

Immunomodulatory effect of chalcones on thrombin-mediated IL-6 and IL-8 production of A549 lung epithelial cells

The chalcone derivatives were studied to determine if they had any immunomodulatory effect on the thrombin-mediated pro-inflammatory cytokines release from A549 cells. A549 cells were seeded at 1.2×10^{5} per well in 24-well cell culture plates and allowed to grow confluent in humidified atmosphere in air at 37 °C, 5% CO_2. The culture medium was replaced with serum-free medium and incubated for 24 h. The A549 cells were then pre-incubated with chalcone derivatives at 100 μM, 50 μM, 10 μM and 1 μM for 2 h and followed by thrombin stimulation (2 units per mL) for 24 h. The cell supernatants were collected and assayed for IL-6 and IL-8 using ELISA (see Fig. 1 and ESI†).

Computer-assisted conformational study of selected chalcones

Computer-assisted conformational studies were performed on selected chalcones (compounds **1**, **3**, and **6–10**) using the program GAUSSIAN03.[5] In a first step the three-dimensional models of the investigated compounds were assembled using the atoms and structural fragments from GAUSSVIEW 3.0. Geometry optimisation was then performed at RHF/3-21G level of theory. After the structures were determined at RHF/3-21G level of theory, partially relaxed scan calculations were run for every 15° rotation of the torsional angles φ and θ at RHF/3-21G level of theory. Energy profiles of compounds **1**,**3**, and **6–10** were obtained at RHF/3-21G level of theory (see ESI†). In order to obtain a better degree of accuracy, the minima obtained for each compound from the relaxed scan calculations were further optimised at RHF/6-31 + G** level of theory, as previous conformational studies on chalcones by Lopez *et al.* showed that results obtained at the RHF/6-31G level of theory were in agreement with experimental X-ray results.[4]

LUMO and MEP maps of selected chalcones

In order to rationalise a possible site of action at the electronic level for compounds **1**, **3**, and **6–10**, we generated LUMO and MEP maps. In a first step, the checkpoint files obtained from

optimisation of these compounds at RHF/6-31+G** were first converted into formatted checkpoint files. The total electron density (density = SCF), electrostatic potential (potential = SCF) and LUMO (MO = LUMO) cube files were then generated from the formatted checkpoint files using the Cubegen utility program in GAUSSIAN03. The number of points per side in the cube was set to the default value of 80 points.[3] MEP surfaces were visualised using GAUSSVIEW 3.0 by mapping the RHF/6-31+G** electrostatic potentials onto the total electron density (isovalue = 0.001 e $Å^{-1}$) (Fig. 2(a)–(g)). LUMO surfaces were visualised by mapping the RHF/6-31+G** LUMO's onto the total electron density (isovalue = 0.001 e $Å^{-1}$) (Fig. 2(h)–(n)). For the MEP maps, regions with attractive potential appear in red and those of repulsive potential appear in blue. For the LUMO maps regions with the highest absolute value of LUMO are indicated in 'blue', while regions with the lowest values are indicated in 'red'.

Acknowledgements

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References

1 J. R. Dimmock, D. W. Elias, M. A. Beazely and N. M. Kandepu, *Curr. Med. Chem.*, 1999, **6**, 1125; M. L. Go, X. Wu and X. L. Liu, *Curr. Med. Chem.*, 2005, **12**, 483; Z. Nowakowska, *Eur. J. Med. Chem.*, 2007, **42**, 125; J. R. Dimmock, A. Jha, G. A. Zello, T. M. Allen, C. L. Santos, J. Balzarini, E. D. Clercq and E. K. Manavathu, *Pharmazie*, 2003, **58**, 227.

2 K. F. Chung and P. J. Barnes, *Thorax*, 1999, **54**, 825; N. Asokananthan, P. T. Graham, D. J. Stewart, A. J. Bakker, K. A. Eidne, P. J. Thompson and G. A. Stewart, *J. Immunol.*, 2002, **169**, 4572.

3 S. H. Lee, G. S. Seo, X. Y. Jin, G. Ko and D. H. Sohn, *Life Sci.*, 2007, **81**, 1535; Y. Liu, C. Hsieh, C. Wu and B. Wung, *Life Sci.*, 2007, **80**, 1420; L. Barfod, K. Kemp, M. Hansen and A. Kharazmi, *Int. Immunopharmacol.*, 2002, **2**, 545; A. Daikonya, S. Katsuki and S. Kitanaka, *Chem. Pharm. Bull.*, 2004, **52**, 1326; O. Nerya, R. Musa, S. Khatib, S. Tamir and J. Vaya, *Phytochemistry*, 2004, **65**, 1389; M. A. Babu, N. Shakya, P. Prathipati, S. G. Kaskhedikar and A. K. Saxena, *Bioorg. Med. Chem.*, 2002, **10**, 4035.

4 S. N. Lopez, M. V. Castelli, S. A. Zacchino, J. N. Dominguez, G. Lobo, J. Charris-Charris, J. C. G. Cortes, J. C. Ribas, C. Devia, A. M. Rodriguez and R. D. Enriz, *Bioorg. Med. Chem.*, 2001, **9**, 1999.

5 M. J. Frisch, G. W. Trucks, H. B. Schlegel, G. E. Scuseria, M. A. Robb, J. R. Cheeseman, J. A. Montgomery, Jr., T. Vreven, K. N. Kudin, J. C. Burant, J. M. Millam, S. S. Iyengar, J. Tomasi, V. Barone, B. Mennucci, M. Cossi, G. Scalmani, N. Rega, G. A. Petersson, H. Nakatsuji, M. Hada, M. Ehara, K. Toyota, R. Fukuda, J. Hasegawa, M. Ishida, T. Nakajima, Y. Honda, O. Kitao, H. Nakai, M. Klene, X. Li, J. E. Knox, H. P. Hratchian, J. B. Cross, V. Bakken, C. Adamo, J. Jaramillo, R. Gomperts, R. E. Stratmann, O. Yazyev, A. J. Austin, R. Cammi, C. Pomelli, J. Ochterski, P. Y. Ayala, K. Morokuma, G. A. Voth, P. Salvador, J. J. Dannenberg, V. G. Zakrzewski, S. Dapprich, A. D. Daniels, M. C. Strain, O. Farkas, D. K. Malick, A. D. Rabuck, K. Raghavachari, J. B. Foresman, J. V. Ortiz, Q. Cui, A. G. Baboul, S. Clifford, J. Cioslowski, B. B. Stefanov, G. Liu, A. Liashenko, P. Piskorz, I. Komaromi, R. L. Martin, D. J. Fox, T. Keith, M. A. Al-Laham, C. Y. Peng, A. Nanayakkara, M. Challacombe, P. M. W. Gill, B. G. Johnson, W. Chen, M. W. Wong, C. Gonzalez and J. A. Pople, *GAUSSIAN 03 (Revision C.2)*, Gaussian, Inc., Wallingford, CT, 2004.