Antioxidant studies on monosubstituted chalcone derivatives - understanding substituent effects

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Abstract: An overproduction of reactive oxygen species beyond basal levels generated continuously in the body as part of natural metabolic processes often results in serious and diverse disease conditions including cancer. Chalcones are known to possess good antioxidant properties, and structure activity relationship studies have been effective in designing molecules with better antioxidant profiles. The present study constitutes a preliminary investigation in seeking safer anti-inflammatory agents with good antioxidant properties. A ten-membered chalcone library - comprising nine monosubstituted derivatives and the unsubstituted parent chalcone - characterized by varying stereoelectronic properties was screened for antioxidant activity using four well established *in vitro* assays including the hydrogen peroxide, nitric oxide and super oxide radical scavenging assays along with reducing power assay. The trends observed were then correlated with their anti-inflammatory profiles. All the derivatives except 4'-phenylchalcone (4i) showed improved antioxidant profiles compared to the unsubstituted parent compound. The three bromo derivatives (4d, 4g and 4j) clearly portrayed the effect that bulky substituents may have on antioxidant activity; with the *para* derivative 4j exhibiting the highest activity amongst these regioisomers. The 2'-hydroxy analog 4b is an optimized lead with the best antioxidant as well as anti-inflammatory properties. There exists a significant correlation between the antioxidant and anti-inflammatory activity.

Keywords: Antioxidants, chalcone, radical scavenging activity and reactive oxygen species.

INTRODUCTION

Chalcones are predominantly phytochemical moieties that serve as intermediates to the flavonoid family of natural products. Previous reports have confirmed numerous bioactivities of chalcone derivatives including anti-inflammatory, antioxidant, neuroprotective and anticancer activity (Beg *et al*., 2011; Chahar *et al*., 2011; Iqbal *et al.*, 2014; Isa *et al*., 2012; Kumar and Khanum, 2012; Prabhakar *et al.*, 2014; Zarghi *et al*., 2006). The promising pharmacological profile of this class of compounds has triggered a renewed interest in further understanding the potential for modifications directed by mechanistic and structure activity relationship (SAR) studies (Cao *et al*., 1997). As illustrated in fig. 1, they are a group of privileged 1,3-diarylprop-2-en-1-ones (1a) wherein the two aromatic rings are linked together with a three-carbon α,β-unsaturated carbonyl group. While the ring directly connected to the ketone is labeled as A ring, the other is referred to as B ring by convention. The general structure along with the numbering scheme for the compounds in this study viz. the 1,3-diphenylprop-2-en-1-ones is represented by 1b.

It has been hypothesized that extended conjugation and enhanced electrophilicity may be among the key reasons for the high bioactivity of this class of compounds (Awasthi *et al*., 2009; Cheng *et al*., 2000). Moreover, the dipoles created in these molecules by inductive and mesomeric effects also contribute to their enhanced bioactivities. Inductive (*+I/-I*) effect occurs when the electron cloud associated with a group of atoms is displaced towards the more electronegative atom. This charge imbalance is transferred through the chain of atoms (through-bond effect) resulting in a permanent dipole. The mesomeric effect represents the electron withdrawing (*-M*) and/or the electron releasing (*+M*) properties of the substituents via the resonance phenomenon. Both the inductive as well as mesomeric effects together play an important role in deciding the electrical properties of a molecule which, in turn, influences its receptor interaction(s) and ultimately bioactivity. The double bond present in these molecules has been known to exist in both the *cis* as well as *trans* geometries with the *trans* configuration proven to be thermodynamically as well as biologically favorable.

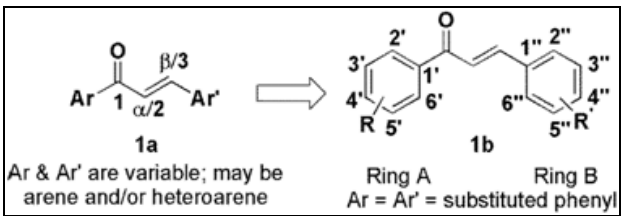

Fig. 1: Chalcone scaffold.

Over the past decade, several modifications to the basic nucleus have led to a better understanding of the chalcone

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scaffold as a pharmacophore. SAR studies have revealed that the α,β-unsaturated ketone moiety is the major pharmacophoric element since its removal leads to loss of activity (Lawrence *et al*., 2006).

Antioxidants are substances that at low concentration prevent or delay oxidation of other molecules (Halliwell and Gutteridge, 1990). All living systems produce reactive oxygen species (ROS) like hydroxyl radicals (OH·), peroxyl radicals (ROO·) and super oxide anion ($O_2^{\cdot-}$) as part of the natural metabolic processes taking place in the system (Halliwell, 1991) and these toxic ROS are counteracted by many natural (proteins, polyamines, retinol, creatinine) as well as dietary (ascorbic acid, carotenoids, flavonoids) antioxidants thereby protecting the cells from oxidative damage (Halliwell and Gutteridge, 1999; Nijveldt *et al*., 2001).The free radical scavenging efficiency of most compounds depends on their overall molecular structure. For example, compounds that can readily donate a hydrogen atom are expected to be good scavengers since the radicals with an unpaired electron are stabilized by the donated hydrogen. Although the biochemical mechanism of radical scavenging might vary across derivatives, the basic principle is to stabilize the radical and prevent it from interacting with endogenous entities and causing damage. Chalcones have been known to possess strong antioxidant properties making them potential candidates against conditions as diverse as cancer and bacterial infections. The main mechanistic hypothesis regarding the antioxidant properties of chalcones is based on the formation of phenoxy radicals owing to the high reactivity of phenolic hydroxyl groups (Kilicgun and Altiner, 2010; Rezk *et al*., 2002). Moreover, the *ortho* and *para* hydroxyl substitutions have been shown to display much superior antioxidant activity compared to that of *meta* substituted ones possibly because of the enhanced stability of the semiquinone radicals that they form (Kim *et al*., 2008). Also, halogenated chalcones have been known to possess a stronger antioxidant potential than their non-halogenated counterparts with bromine and fluorine derivatives preferred over that of chlorine substituents (Isaac *et al*., 2012).

It is well established that oxidative damage and inflammatory conditions have strong links (Lazer *et al*., 1989; Pashkow, 2011; Schinella *et al*., 2002). For this reason, the design of antioxidants with anti-inflammatory activity has been a very interesting field. The ROS generated as part of normal metabolic processes trigger the activation of many redox sensitive signaling pathways out of which tyrosine kinase and mitogen activated protein kinase (MAPK) play crucial roles in transcriptional activation (Chen *et al*., 2006; Chung *et al*., 2011). They may also be involved in various downstream signaling pathways that generate transcription factors which bind to DNA/RNA in the nucleus leading to the production of inflammatory mediators (adhesion molecules, chemokines, and pro-inflammatory genes) (Lin *et al*., 2005). From this hypothesis, it is clear that suppression of ROS by antioxidants can contribute significantly towards the inhibition/control of inflammatory response. Here we discuss the synthesis and evaluation of a series of monosubstituted A ring chalcone derivatives whose antioxidant properties exhibit very good correlation with their anti-inflammatory activity.

MATERIALS AND METHODS

Chemicals and standards

All chemicals used were of analytical grade unless specified otherwise. The precursor compounds were purchased from commercially available sources. 2'-Bromo acetophenone (99%, Sigma Aldrich, UK), 3'-bromo acetophenone (98%, Alfa Aesar, UK), 4'-bromo acetophenone (98%, Otto Kemi, India), 2'-hydroxy acetophenone (98%, Otto Kemi, India), 4'-methoxy acetophenone (98%, Otto Kemi, India), 4'-phenyl acetophenone (98%, Alfa Aesar, UK), 2'-methyl acetophenone (98%, Alfa Aesar, UK), 2'-amino acetophenone (96%, Otto Kemi, India), 2'-nitro acetophenone (98%, Otto Kemi, India), *n*-naphthylethylenediamine dihydrochloride (98%, Sigma Aldrich, UK), nitro blue tetrazolium (98%, TCI Chemicals, India), ascorbic acid (99.5%, Spectrum Reagents and Chemicals, India).

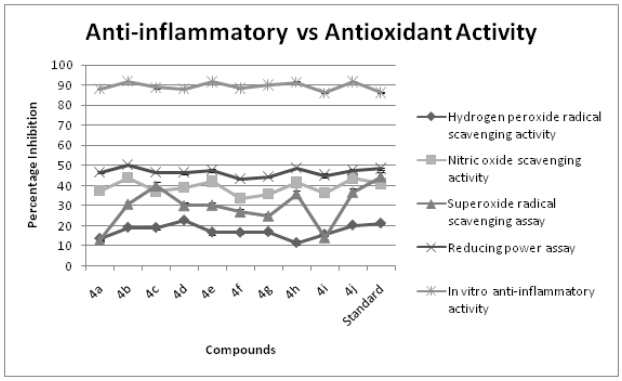


Fig. 2: A comparison of the *in vitro* antioxidant and anti-inflammatory activity of the synthetic chalcone derivatives at 20µg/ml. Data represented as mean±SD (n=3). Standard for antioxidant assay = ascorbic acid. Standard for anti-inflammatory assay = ibuprofen.

Design and synthesis

Ten derivatives were conceived with the parent chalcone serving as the base compound. The substituents were selected based on their electron withdrawing and electron releasing ability, both via inductive as well as mesomeric effects. The target compounds included: unsubstituted chalcone (4a), 2'-hydroxychalcone (4b), 2'-methylchalcone (4c), 2'-bromochalcone (4d), 2'-aminochalcone (4e), 3'-nitrochalcone (4f), 3'-bromochalcone (4g), 4'-methoxychalcone (4h), 4'-phenylchalcone (4i) and 4'-bromochalcone (4j). This

focused library was designed in such a way that the constituent members listed above contained a 2'- (*ortho*), 3'- (*meta*) or 4'- (*para*)-substituted A ring while their B ring was left unsubstituted. The amino, hydroxy, methoxy and nitro groups were selected for their mesomeric (*+M/-M*) effects while the methyl and bromo groups for their inductive (*+I/-I*) effects. The phenyl substitutent was included to probe the putative role of sterics in pharmacological activity. The design considerations and some *in silico* criteria like drug-likeness of a similar larger series of chalcones have been discussed at length in an earlier publication (Balasubramanian and Vijayagopal, 2012).

Synthesis of the target chalcones was achieved using the Claisen-Schmidt reaction, which is a variant of the classic base-catalyzed aldol condensation (Scheme 1). Standard spectroscopic techniques including ^{1}H NMR, ^{13}C NMR, FTIR and mass spectrometry were used to confirm the structures of the synthesized compounds. Detailed synthetic procedures, physicochemical properties and spectral characterization of the derivatives have been described elsewhere (Balasubramanian *et al.*, 2013).

Hydrogen peroxide scavenging activity

The ability of the synthetic compounds to scavenge the hydrogen peroxide free radicals was measured using the method of Ruch (Ruch *et al*., 1989; Sivakumar *et al*., 2011). 0.3ml of 30mM hydrogen peroxide solution was added to 400µl of chalcone and incubated for 10min after which the absorbance was measured spectrophotom-etrically at 238nm against a blank of phosphate buffer devoid of hydrogen peroxide. All spectral analysis was performed using a UV-1800 Shimadzu UV-Vis double beam spectrophotometer (Shimadzu, Japan). The percentage scavenging activity for both the tests as well as the standards was determined. The assays were carried out in triplicate and results expressed as mean±standard deviation (SD).

$$\%\ \text{hydrogen peroxide scavenging activity} = \frac{Ac - As}{Ac} \times 100$$

, where Ac = absorbance of control and As = absorbance of test sample.

Nitric oxide radical scavenging activity

Nitric Oxide was spontaneously generated from sodium nitroprusside and measured by the Griess reagent (Marcocci *et al*., 1994). A mixture of 0.5ml of sample, 0.5ml of phosphate buffer (pH 7.4) and 2ml sodium nitroprusside solution was incubated at 25°C for 2h. 0.5ml of this reaction mixture was allowed to react with 1ml of sulphanilic acid for 5min to complete the diazotization reaction. To this mixture, 1ml of 0.1% *n*-naphthylethylenediamine dihydrochloride was added, and allowed to stand for 30min to form pink coloured chromophore. Absorbance was measured at 530nm against the corresponding blank solution. The absorbance of ascorbic acid as positive control was also determined using the same procedure. The difference in the absorbance between test and control was calculated and expressed as percent scavenging of nitric oxide radical. The assays were carried out in triplicate and results expressed as mean±SD.

$$\%\ \text{nitric oxide scavenging activity} = \frac{Ac - As}{Ac} \times 100$$

, where Ac = absorbance of control and As = absorbance of test sample.

Superoxide radical scavenging activity

The assay is based on the capacity of test compounds to inhibit the reduction of NBT in the riboflavin-light-NBT system (Martinez *et al*., 2001; Sivakumar *et al*., 2011). 3 ml of reaction mixture was prepared containing 50mM of sodium phosphate buffer (at a pH of 7.8), 13mM methionine, 2µM riboflavin, 100µM EDTA, 75µM NBT and 1ml of 50µg/ml test sample. The increase in absorbance at 560nm, after 10min of illumination from a fluorescent lamp, due to the formation of blue formazan was measured. The assays were carried out in triplicate and results expressed as mean±SD. The activity was determined as follows:

$$\%\ \text{superoxide scavenging activity} = \frac{Ac - As}{Ac} \times 100$$

, where Ac = absorbance of control and As = absorbance of test sample.

Reducing power assay

The method of Sivakumar (Sivakumar *et al*., 2011) was used to determine the reducing power of the test compounds. After preparing various concentrations of chalcones in methanol, 2.5ml of sodium phosphate buffer (0.2M, pH 6.5) and 2.5ml of 1% potassium ferricyanide were added to each of these methanolic solutions. Each mixture was incubated at 50°C and after adding 2.5ml of 10% (w/v) trichloroacetic acid, the mixture was centrifuged at 3000rpm for 10min. To 2.5ml of supernatant, 2.5ml of deionized water and 0.5ml of 0.1% ferric chloride were added and the absorbance measured at 700nm. The assays were carried out in triplicate and results expressed as mean±SD. The reducing power of the test compound and standard ascorbic acid is determined as follows:

$$\%\ \text{reducing power} = \frac{Ac - As}{Ac} \times 100$$

, where Ac = absorbance of control and As = absorbance of test sample.

STATISTICAL ANALYSIS

All the antioxidant assays described herein were carried out in triplicate and results expressed as mean±SD. All statistical analysis was performed using Graph Pad (Version-3) Prism software. One-way ANOVA was used to test the differences between IC_{50} values of the different derivatives in the four assays followed by post hoc Tukey's multiple comparison test and $P < 0.001$ was considered statistically significant.

RESULTS

The ability of the test compounds to act as antioxidants was assessed by computing their IC_{50} values. In the context of this study, an estimated IC_{50} value associated with a compound for a given antioxidant assay (out of the four conducted here) represents the concentration of that compound which is capable of inhibiting the specific *in vitro* chemical reaction under consideration by 50%. The IC_{50} values of the chalcone derivatives ranged from 25-95 µg/ml across all the four assays clearly indicating a strong free radical scavenging activity of the synthesized compounds. While the lowest IC_{50} values were recorded for the reducing power assay, the highest values were observed for the hydrogen peroxide radical scavenging assay reflecting varying sensitivities of the compounds towards different assays (table 1). Moreover, the test compounds exhibited different degrees of activity between the individual assays. However, among the ten compounds screened, 2'-hydroxychalcone (4b) exhibited the lowest IC_{50} value consistently throughout all the assays indicating good activity. Analogs 4c, 4f, 4h and 4j were found to have the highest IC_{50} in the hydrogen peroxide radical scavenging activity, while 4h had the highest IC_{50} (67µg/ml) in the nitric oxide radical scavenging activity. In the super oxide radical scavenging assay, 4h exhibited significant radical quenching comparable to that of the standard drug ascorbic acid. In the reducing power assay, 4b was found to have a very good antioxidant activity (IC_{50}: 25µg/ml) that was much stronger than the standard while 4h had the least activity.

The three bromo derivatives (4d, 4g and 4j) exhibited significant variations in the antioxidant activities based on their point of attachment in the A ring. Keeping all other factors constant and taking a simplified view of steric considerations, it would be reasonable to expect the *ortho* congener to fare the worst amongst a bulky series of regioisomeric compounds.

After assessing the percentage inhibition of the ten target chalcones (data not shown) in all four antioxidant assays performed above at the five dilutions (20, 40, 60, 80 and 100µg/ml in each case), a comparison of this data with the results of the anti-inflammatory assays communicated by our group elsewhere (Balasubramanian *et al.*, 2013) has been illustrated in fig. 2.

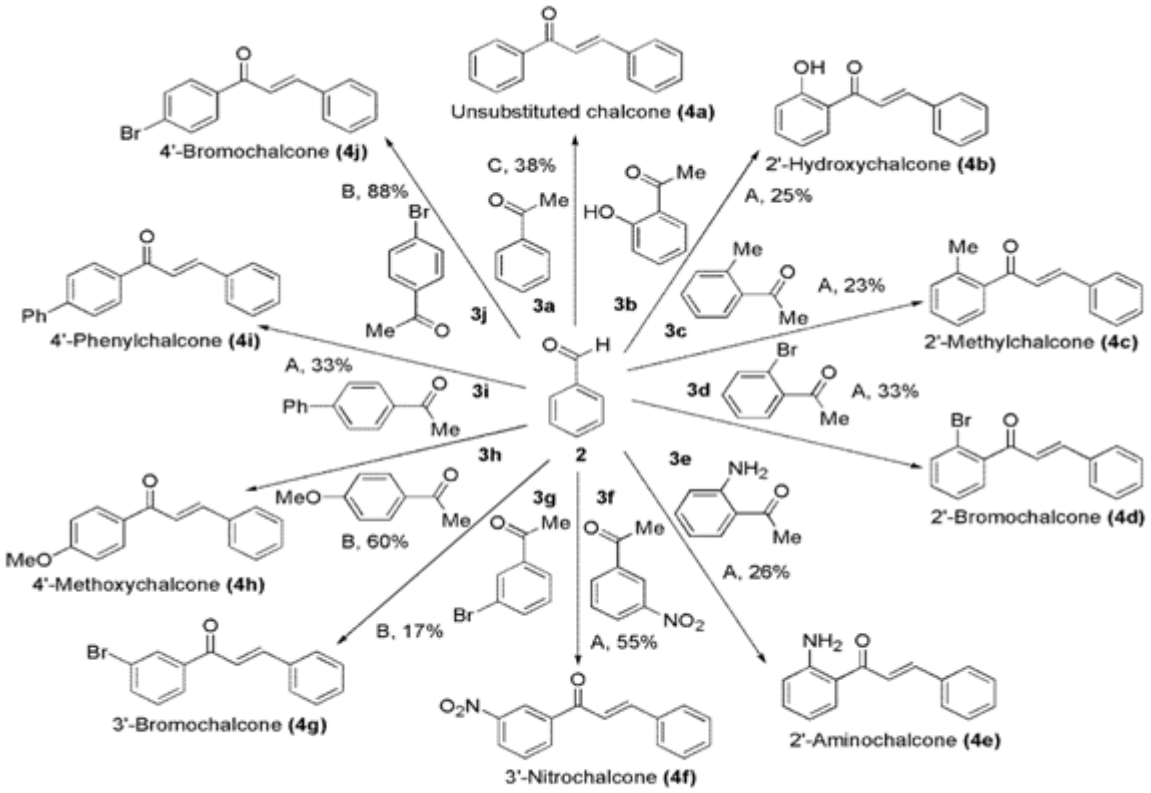
DISCUSSION

The antioxidant properties of chalcones have been well corroborated in this study. From the diverse antioxidant profiles of the congeners evaluated herein, it is evident that substituents indeed have a profound effect on activity. There is an overall trend in antioxidant activity across the four assays performed with the 2'-hydroxy derivative (4b) emerging as the most active one consistently. However, many derivatives show characteristic profile in specific assays, indicating a significant free radical scavenging activity ($P < 0.001$) of the synthesized library. Since the results from the nitric oxide radical scavenging assay were more consistent and correlated better with the anti-inflammatory activity (fig. 2), this data was used in the ensuing interpretation.

From the overall antioxidant profile, it is evident that the *meta* substituents were detrimental for optimal activity, while the *ortho* and the *para* positions seem to be favorable. Furthermore, among the *ortho* congeners the hydroxyl and amino derivatives (4b and 4e) were much more active probably because of their mesomeric (*+M*) effects. However, the methyl and bromo derivatives (4c and 4d) with their associated inductive (*+/-I*) effects were much less active. Among the two *meta*-substituted derivatives 3'-nitrochalcone (4f) and 3'-bromochalcone (4g), the latter was found to be more active via its electron withdrawing inductive effect (*-I*) compared to the nitro derivative (*-M*). Within the three *para*-substituted compounds in this study, the electron releasing *+M* methoxy substituent possesses good nitric oxide radical scavenging activity. This leads us to summarize that substituents on the A ring that release electrons via mesomeric effect may be favorable to antioxidant activity irrespective of their regiochemistry.

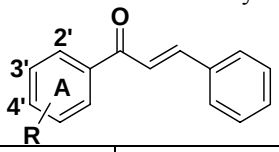
In confirmation with previous studies (Anto *et al*., 1995), the hydroxyl derivative 4b was found to be superior compared to the rest of the analogs screened. More significantly, previous reports precisely suggest that the *ortho* hydroxy derivative is more active compared to the corresponding *para* and *meta* isomers probably because of the more efficient stabilization of the phenoxy radicals that is possible at the *ortho* position (Velika and Kron, 2012).

The phenyl derivative 4i was the least active compound presumably because of the extensive steric effects exerted by the large, hydrophobic phenyl group. Recent work in our laboratory revealed that the phenyl derivative was an outlier with respect to the partition coefficient value (MLogP = 5.93) during a routine *in silico* screen to check compliance of compounds with Lipinski's rule of five (Balasubramanian and Vijayagopal, 2012). High lipophilicity associated with such greasy molecules will favor their preferential partitioning and subsequent entrapment in the lipid phase preventing them from exerting their protective radical scavenging action, thereby causing a deleterious effect on their antioxidant potential. Given the fairly large size of the bromine atom, the three bromo derivatives 4d, 4g and 4j have served as probes for evaluating the effect of bulkiness and regiochemistry of substituents on the antioxidant profile of this library of chalcones. As expected, the *ortho* isomer showed the highest IC_{50} values signifying the detrimental effect of bulky substituents on antioxidant property.



Scheme 1: Synthesis of chalcone derivatives 4a-4j. All ten target compounds were prepared via a single step condensation between benzaldehyde (2) and appropriately substituted acetophenones (3a-j). The yields of the products obtained ranged from 17-88%. With the exception of the parent moiety 4a that was accessed by microwave irradiation of the reactants, the other analogs were synthesized by conventional solution phase methods employing either sodium or potassium hydroxide as the base.

Table 1: IC_{50} of chalcone derivatives in various *in vitro* antioxidant systems.



Compound	R	Hydrogen peroxide radical scavenging assay	Nitric oxide scavenging assay	Superoxide radical scavenging assay	Reducing power assay
4a	H	88.03±0.09[a]	47.15±0.37[a]	93.04±0.15[a]	32.23±0.29[a]
4b	2'-OH	81.07±0.50[c]	33.98±0.17[e]	57.12±0.31[b]	24.66±0.63[b]
4c	2'-CH_3	93.78±0.55[b]	50.87±0.13[b]	91.79±0.18[a]	37.55±0.74[c]
4d	2'-Br	86.50±0.39[a]	54.53±0.56[f]	77.40±0.45[c]	35.53±0.47[c]
4e	2'-NH_2	89.33±0.47[a]	42.52±0.76[c]	69.95±0.19[e]	29.20±0.30[a]
4f	3'-NO_2	91.76±0.55[b]	43.57±0.37[c]	87.22±0.28[f]	30.49±0.08[a]
4g	3'-Br	81.16±0.98[c]	38.41±1.09[d]	58.72±0.35[b]	31.20±0.33[a]
4h	4'-OCH_3	93.35±0.32[b]	66.65±0.50[g]	38.53±0.52[d]	38.98±0.02[c]
4i	4'-Ph	85.36±0.32[a]	46.49±0.70[a]	50.66±0.57[g]	32.30±0.71[a]
4j	4'-Br	94.16±0.83[b]	51.02±0.15[b]	78.29±0.26[c]	35.67±0.40[c]
Ascorbic acid	NA	77.31±0.45[d]	37.42±0.47[d]	38.38±0.44[d]	26.12±0.29[b]

Note: Values expressed as mean±SD (n=3). IC_{50} was calculated from concentration/response regression line in compounds with more than 50% inhibition, and a range of 5 concentrations were used. All values expressed in µg/ml. Means not sharing the same letters are significantly different at $P < 0.001$ in each column. NA = Not applicable.

Interestingly, it is to be noted that all substitutions have enhanced the antioxidant activity compared to the unsubstituted parent chalcone moiety indicating that ring A would be a good choice for SAR manipulations towards improving antioxidant profiles of chalcone derivatives. While the most optimal antioxidant leads were the 2'-hydroxy derivative (4b) and 2'-amino derivative (4e), 4'-phenylchalcone (4i) turned out to be the least active molecule in this series.

Of even greater significance is our observation of the close overlap of antioxidant profiles of the molecules tested with their anti-inflammatory activity (fig. 2). Remarkably, the best and worst anti-inflammatory and antioxidant compounds turned out to be identical in both cases. Besides, a strikingly similar SAR profile can be inferred leading us to suggest that with the exception of the phenyl derivative all other substitutions generally displayed favorable effects over the unsubstituted derivative with respect to both activities. More specifically, the *ortho* derivatives exhibit much better activity compared to both the *meta* and *para* derivatives.

CONCLUSIONS

The possibility of simultaneously probing the SAR of the chalcone scaffold with respect to both antioxidant as well as anti-inflammatory activity profile was the motive behind this work. *In vitro* antioxidant evaluation of a series of ten A ring monosubstituted chalcones was carried out and the observations from this investigation were correlated with the anti-inflammatory activity of these derivatives. This preliminary screening leads to the conclusion that isolated single substituents on the A ring are not detrimental to overall antioxidant potential. Moreover, the *ortho* derivatives were identified as being the most suitable for antioxidant activity. Bulky hydrophobic groups were found to be suboptimal with the phenyl derivative 4i exhibiting the least activity. Substituent regiochemistry was also found to play a role in bioactivity as is evident from the profile of the three bromo derivatives. Compound 4b with the small hydrophilic substitution on the A ring emerged as the optimized lead with significant antioxidant as well as anti-inflammatory activity. The parallel structure function trends on display between the two activities is very encouraging and has paved the way for more efficient medicinal chemistry efforts to gain a better understanding of the structural requirements of chalcones in the context of inflammation.

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