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In Vitro Anticancer Activity of Monosubstituted Chalcone Derivatives

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Abstract Cancer is a continuously adapting dynamic condition for which investigation of novel treatment strategies constitutes an integral component in its management. As part of the continuing efforts from the global community directed at eradication of cancer, we were interested in evaluating the cytotoxic potential of a series of chalcone derivatives. The study also contributes towards a preliminary understanding of the structure-activity relationship of synthetic chalcone derivatives. We screened various A-ring monosubstituted chalcone derivatives against five selected human cancer cell lines using the sulforhodamine B assay. Our in vitro observations indicated the previously established characteristic biphasic response of the chalcones. Moreover, it was found that the different substitutions at the A-ring were found to suppress the cytotoxic activity, which was evident from higher growth suppression by the unsubstituted chalcone compared to the substituted derivatives. The compound **4c** exhibited similar activity to that of the unsubstituted derivative, indicating that the methyl group is not detrimental to activity. It was observed that the tested compounds were most effective on the A2780 ovarian cancer cell line exhibiting GI_{50} value ranging from 20-66 μM. Moreover, the analogs proved to be least cytotoxic in the HepG2 cell line with GI_{50} values that ranged from 39-100 μM. This study may act as a preliminary step towards understanding substituent effects on the cytotoxic activity profiles of chalcone derivatives emphasising on A-ring substituted moieties.

Keywords Chalcones, Cytotoxicity, Cancer Cell Lines, Sulforhodamine B assay

1. Introduction

A large number of anticancer drugs currently in clinical practice are either plant-derived or synthetic derivatives of plant metabolites, including clinically significant classes of cytotoxic compounds like taxols, vinca alkaloids, and other similar molecules. Chalcones, which constitute a privileged group of compounds, are biosynthetic precursors of naturally occurring flavonoids and isoflavonoids found in a variety of plant species. They possess a 1,3-diaryl-prop-2-ene-1-one scaffold in which the two aromatic rings (labelled A and B) are linked by an α,β-unsaturated carbonyl system (Figure 1). Quercetin, xanthohumol, isoxanthohumol, genistein, naringenin and chalconaringenin are some of the well known pharmacologically active members of this class of natural products. Specific examples of antiproliferative chalcones found in nature include isoliquiritigenin, isobavachalcone, xanthoangelol and licochalcone A[1]. Extended conjugation and a high degree of electrophilicity associated with the moiety play an important role in the activity of this class of compounds[2-4]. The complete delocalization of π electrons on both the benzene rings makes these compounds more susceptible in undergoing electron transfer reactions that may be attributed to their good antioxidant activity. It has been indicated that the *s-trans* conformation is more active in demonstrating cytotoxic activity than the *s-cis* conformation [3].

The extremely simple skeleton of chalcone compared to the complex structures of most other anticancer drug candidates makes this scaffold very attractive to chemists for alteration and structure-activity relationship (SAR) modifications. The diverse pharmacological activity of this class of compounds has drawn the attention of medicinal chemists and pharmacologists alike for a long time. Chalcone derivatives have shown anticancer, antimalarial, antiprotozoal, anti-inflammatory, antibacterial, antifilarial, antifungal, antimicrobial, anticonvulsant and antioxidant

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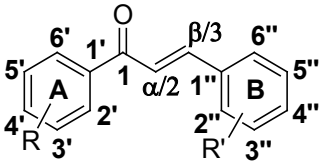
activity[5-14]. The anticancer activity of chalcone derivatives has been a topic of interest due to the association of specific functional groups and substituents to a particular activity or lack thereof against a specific cell line. For instance, the radical quenching activity has been firmly associated with the phenolic hydroxy groups[15-17].



R & R' are variable; 1,3-diaryl-prop-2-ene-1-ones **(1)**

Figure 1. Basic chalcone skeleton with substituted rings A and B

One of the most widely studied antiproliferative mechanisms of action of chalcone derivatives is microtubule destabilization that prevents the polymerization of tubulin protein to form microtubules that are essential for mitotic activity[18, 19]. SAR studies have confirmed that chalcone derivatives with electron-donating hydroxy or methoxy groups act in this fashion. Chalcones bind to the colchicine binding site on the β subunit of tubulin, thus triggering depolymerization[20, 21]. Chalcone derivatives have been found to be significantly active in growth inhibition and induction of apoptosis in prostate cancer cell lines. Besides, they have also been studied for their chemopreventive effects by sensitizing the cells to TRAIL TNF[(Tumor Necrosis Factor)-related apoptosis-inducing ligand]-mediated apoptosis[21, 22]. Studies have indicated that the cytotoxicity of chalcones is related linearly to their ability to form phenoxy radicals by oxidation, as well as to their hydrophilicity[23]. In particular, the cytotoxic mechanisms of the hydroxy derivatives were attributed to the mitochondrial uncoupling and subsequent interference with the cellular respiration[24]. Apart from the apoptosis phenomenon, many other possible mechanisms like inhibition of tubulin assembly, inhibition of angiogenesis, anti-estrogenic activity, and reversal of multi drug resistance (MDR) have been attributed to the anticancer activity of this chosen class of compounds[15, 16]. The present study was carried out on a mini selection of monosubstituted chalcones in order to get a preliminary understanding of the role of substituents, with respect to their electronics as well as regiochemistry, on the antitumor activity of the chalcone skeleton.

2. Materials and Methods

2.1. Reagents and Chemicals

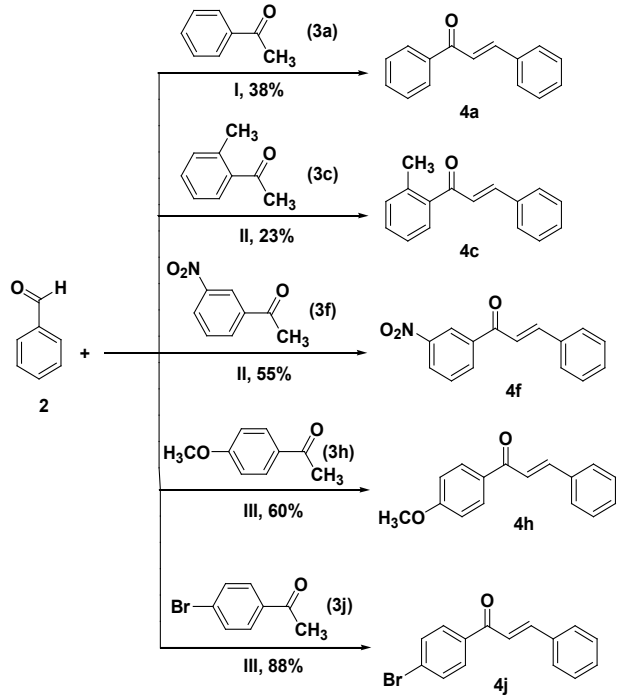
$NaHCO_3$ (Sigma), 10 mM minimal essential medium (MEM) non-essential amino-acid solution (Invitrogen), 100 mM sodium pyruvate (Hyclone), FBS (PAA Laboratories), MEM (Eagle) supplemented with 2 mM L-glutamine and Earle's balanced salt solution (MEM/EBSS; Hyclone), 10 mg/mL bovine insulin in 25 mM HEPES, pH 8.2 (Sigma), 2.5% (wt/vol) trypsin solution (Invitrogen), 0.5% (wt/vol) phenol red solution (Sigma), 0.48 mM versene-EDTA, 0.4% (wt/vol) trypan blue in 0.81% (wt/vol) NaCl and 0.61% (wt/vol) KH_2PO_4 (Sigma), dimethyl sulfoxide (DMSO; Sigma), 10% (wt/vol) trichloroacetic acid (TCA), 10 mM unbuffered Tris base solution.

2.2. Design and Synthesis of Chalcone Derivatives

Five derivatives were carefully designed based on medicinal chemistry principles. Since the objective of the study was to understand substituent effects on the activity, the unsubstituted chalcone (**4a**) formed the base compound. In order to realize the stated goal, the plan was to append substituents of different chemical nature at each of the three available regioisomeric positions on the A-ring. The task of choosing the substituents involved identifying functional groups that withdraw as well as release electrons both by inductive and mesomeric effects. The target derivatives included 2'-methylchalcone (**4c**), 3'-nitrochalcone (**4f**), 4'-methoxychalcone (**4h**), and 4'-bromochalcone (**4j**). While the alkyl and halo groups in **4c** and **4j** release and withdraw electrons inductively (+*I* and –*I* respectively), the nitro and alkoxy groups in **4f** and **4h** do so via resonance (-*M* and +*M* respectively). Regiochemistry was a crucial design consideration as these four derivatives comprising of an *ortho* (represented by carbon atoms 2' and 6' in Figure 1) congener, a *meta* (represented by carbon atoms 3' and 5' in Figure 1)-substituted chalcone and two *para* (represented by carbon atom 4' in Figure 1) analogs account for all the available positions in the A-ring. These four monosubstituted chalcones together with the parent moiety **4a** complete the set of target compounds in this study. Since this work is hoped to serve as a starting point for systematic SAR investigation, five additional chalcone derivatives [2'-hydroxychalcone (**4b**), 2'-bromochalcone (**4d**), 2'-aminochalcone (**4e**), 3'-bromochalcone (**4g**), and 4'-phenylchalcone (**4i**)] were prepared following the same design criteria. Based on the preliminary findings reported herein, all these ten analogs would be subjected to further in depth studies in order to facilitate greater understanding of structure function correlation.

The target chalcones were readily accessed via a single step reaction with varying yields as illustrated in Scheme 1. Called the Claisen-Schmidt condensation, this fundamental reaction between benzaldehyde **2** and either acetophenone (**3a**) or suitably substituted acetophenones (**3c**, **3f**, **3h** and **3j**) is a procedural variant of the classic base-catalyzed aldol condensation reaction. Although the parent chalcone **4a** was rapidly assembled by microwave irradiation of the reactants for about 8 minutes, it was only obtained in a 38% yield. The other analogs were prepared in much higher yields of up to 88% by routine solution phase methods using either sodium or potassium hydroxide as the alkali. It is pertinent to note here that the commercial availability of the corresponding acetophenones was an important criterion which dictated the

choice of A-ring substituents. Characterization of these derivatives was then carried out by standard spectroscopic techniques including ^{1}H NMR, ^{13}C NMR, FTIR and mass spectrometry. The detailed synthetic protocols, physico - chemical properties of the synthesized analogs and the assignment of the relevant spectral signals and/or bands have been described elsewhere[Balasubramanian, R., Iqbal, H., Vijayagopal, R., and Baby, C., Design, synthesis and evaluation of a ring-A monosubstituted chalcone library for anti-inflammatory activity., Indian J. Pharm. Educ. Res., 'under review'].



I, II & III represent the procedural variants of the reaction used.
I: Conc. aq. KOH, rectified spirit, 160 W microwave irradiation;
II: 10% aq. KOH, rectified spirit, rt, 16 h; III: 10% aq. NaOH, rectified spirit, rt, 5.5 h

Scheme 1. Synthesis of target chalcone derivatives

2.3. Cell Culture and In Vitro Cytotoxicity Assay

Cytotoxicity of the five compounds was estimated in different human cell lines using the sulforhodamine B (SRB) protocol[25]. Five human cell lines - U373MG (human glioblastoma cell line), MCF-7 (human breast cancer cell line), HepG2 (human pancreatic cancer cell line), K562 (human myelogenous leukemia cell line) and A2780 (human ovarian cancer cell line) were selected for this study. Cells were inoculated into 96-well microtiter plates (90 µL/well) at appropriate plating densities depending on the doubling time of individual cell lines. The microtiter plates were incubated at 37 °C in a carbon dioxide (CO_2) incubator at 5% CO_2, 95% air and 100% relative humidity for 24 h prior to addition of experimental drugs. The cells were then incubated for 48 h with the test compounds. The experiment was terminated by adding 30% chilled TCA to the wells. Cells were stained using 0.4% SRB in 1% acetic acid. The SRB dye bound to the fixed cells was eluted using 10 mM unbuffered Tris solution. The optical density was measured using ELISA micro plate reader (Tecan-Sunrise) at a wavelength of 540 nm with 690 nm reference wavelength. Percent growth was calculated on a plate-by-plate basis for test wells relative to control wells and was expressed as the ratio of average absorbance of the test well to the average absorbance of the control wells multiplied by 100. Using the six absorbance measurements[time zero (Tz), control growth (C), and test growth in the presence of drug at the four concentration levels (Ti)], the percentage growth was calculated at each of the drug concentration levels. Percentage growth inhibition was computed using the formulae:

$$[(Ti-Tz)/(C-Tz)] \times 100 \text{ for concentrations wherein } Ti \geq Tz \text{ i.e., } (Ti-Tz) \text{ positive or zero} \quad (1)$$

$$[(Ti-Tz)/Tz] \times 100 \text{ for concentrations wherein } Ti < Tz \text{ i.e., } (Ti-Tz) \text{ negative} \quad (2)$$

The dose response parameters were then calculated for each test compound. Growth inhibition of 50% (GI_{50}) was calculated from$[(Ti-Tz)/(C-Tz)] \times 100 = 50$, which is the drug concentration resulting in a 50% reduction in the net protein increase (as measured by SRB staining) in control cells during the drug incubation. The drug concentration resulting in total growth inhibition (TGI) was calculated from $Ti = Tz$. The LC_{50} (concentration of drug resulting in a 50% reduction in the measured protein at the end of the drug treatment as compared to that at the beginning) that is indicative of a net loss of cells following treatment is calculated from$[(Ti-Tz)/Tz] \times 100 = -50$. Values were calculated for each of these three parameters if the level of activity was reached; however, if the effect was not reached or was exceeded, the values for that parameter were expressed as greater or less than the maximum or minimum concentration tested.

2.4. Statistical Analysis

All values were expressed as mean ± standard error of mean (SEM). All statistical analysis was performed using Graph Pad (Version-3) Prism software. Data for cytotoxicity studies were analyzed using one way Analysis of Variance (ANOVA) followed by Dunnetts test. A confidence interval of 95% was selected and $P<0.05$ was considered statistically significant compared to the control group.

3. Results and Discussion

3.1. Cytotoxic Parameters

The results of the cytotoxicity assay exhibit an interesting trend across all the cell lines. Almost all the chalcone derivatives were found to be active only at 10^{-4}M concentration. Moreover there is no linear response between the drug concentration and cell death. While the 10^{-4}M concentration exhibited significant activity, the 10^{-5}M concentration did not exhibit an activity that was even comparable to the former. The same trend was found in the other higher dilutions also. This interesting phenomenon is in conformation with a previously reported study that

attributes this trend to the characteristic biphasic response of chalcone derivatives[26]. In the case of the U373MG cell lines, **4h** was found to be the least active while **4a** exhibited highest cytotoxicity. It is evident that the compounds **4c** and **4j** exhibited higher cytotoxicity than the reference compound Adriamycin (Figure 2). In MCF-7 breast cancer cell line, derivatives **4a** and **4c** exhibited the highest activity; however, consistent with the previous neuronal cell line, the cytotoxic activity showed a significant increase at 10^{-4} M concentration (Figure 3).

In case of the human pancreatic cancer cell line HepG2, all five compounds exhibited very less activity in the first three concentrations but the 10^{-4} M concentration range produced strong cytotoxicity in three of the compounds, viz. **4a**, **4c** and **4j** (Figure 4). Compounds bearing the 3'-nitro and 4'-methoxy substituents (**4f** and **4h** respectively) showed negligible cytotoxicity in this cell line. Yet again, the 2'-methyl substituent was seen eliciting higher cytotoxicity than the reference compound. The human leukemia cell line K562, also displayed a similar activity profile wherein the first three concentrations exhibited the same negligible cytotoxic activity, irrespective of the A-ring substituent (Figure 5). In the 10^{-4} M concentration range, there was an increase in the cytotoxic activity, with the 3'-nitro derivative **4f** showing the least activity and the unsubstituted chalcone **4a** the highest activity. None of the compounds exhibited a comparable activity with the reference drug in this particular cell line.

The biphasic trend on display with regard to the cytotoxicity of the compounds was once again observed in A2780 ovarian carcinoma cell line. The first three concentrations exhibited almost similar and negligible activity, but the fourth dilution resulted in sudden dip in the percentage cell growth (Figure 6). The 2'-methylchalcone **4c** exhibited the highest activity in this particular cell line. Among the five cell lines evaluated in this study, all the compounds showed the least total growth inhibition in the human leukemia cell line.

In the context of growth inhibition, Adriamycin always showed a linear cytotoxic response with respect to drug concentration. On the other hand, chalcones under consideration in this study seem to possess anti-proliferative activity only at higher concentrations (~100 μM) with no effect on cell growth at lower concentrations in the range of 0.1-10 μM. It is appropriate to emphasize here that this result is not an experimental artefact and that it did not arise due to any technical irregularity in the assay protocol/methodology adopted or due to any statistical discrepancy in the result. This observation therefore clearly indicates that a biphasic response is on display in the tested derivatives and corroborates earlier findings of similar nature in the context of anti-proliferative activity[26].

A study of the GI_{50} values on the different cell lines indicates that the tested compounds were most effective on the ovarian cancer cell line A2780, with the GI_{50} values ranging from 20-66 μM, while the least sensitive cell line was found to be HepG2 with the GI_{50} values ranging from 39-100 μM (Table 1). In the human CNS cancer cell line, the GI_{50} values ranged from 34-82 μM. Among the five tested compounds, **4a** and **4c** were the most effective against U373MG cell line. In case of the MCF-7, **4a** was found to be the most active while the 2'-methyl derivative **4c** was the most potent against the pancreatic cancer cell line. The GI_{50} values for the leukemia cell line ranged from 30-74 μM. The parent chalcone **4a** was once again the most active compound against this cell line while **4c** was found to be the most potent derivative against the human ovarian cancer cell line. The human breast cancer cell line exhibited intermediate GI_{50} values, ranging from 27-52 μM.

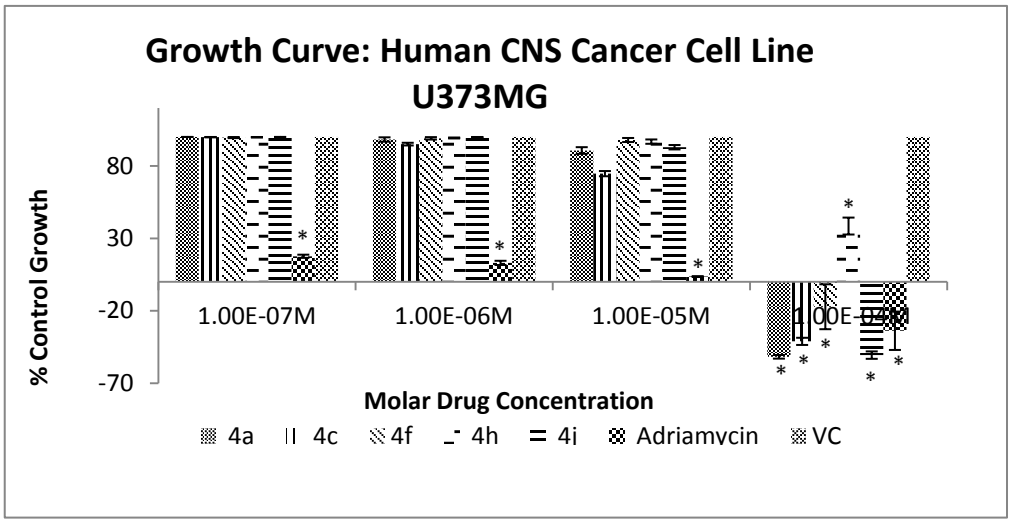


Figure 2. Effect of different chalcone derivatives on the percentage growth inhibition of the U373MG cell line[Values are Mean ± SEM of 3 replicates each. *P<0.05, VC = Vehicle Control (DMSO)]

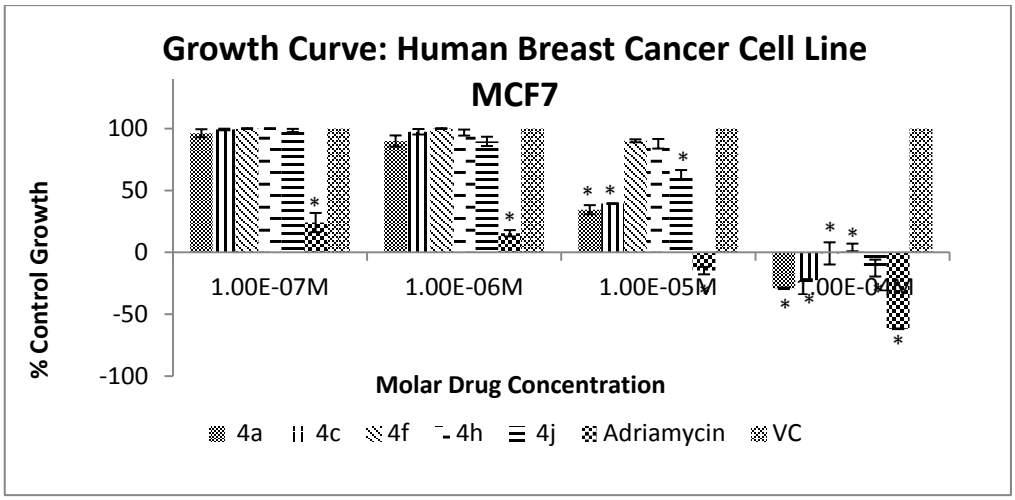


Figure 3. Effect of different chalcone derivatives on the percentage growth inhibition of the MCF-7 cell line[Values are Mean ± SEM of 3 replicates each. *P<0.05, VC = Vehicle Control (DMSO)]

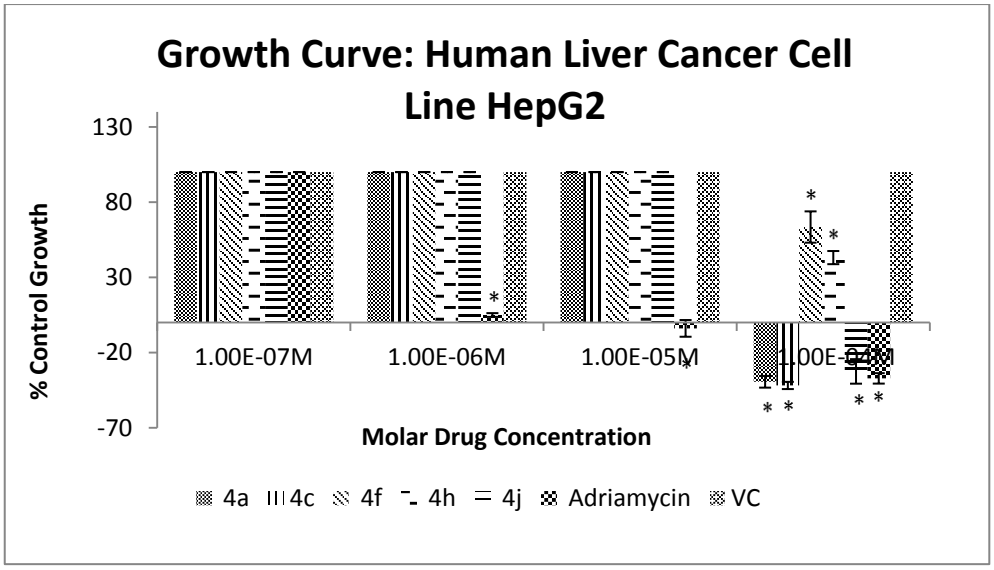


Figure 4. Effect of different chalcone derivatives on the percentage growth inhibition of the HepG2 cell line[Values are Mean ± SEM of 3 replicates each. *P<0.05, VC = Vehicle Control (DMSO)]

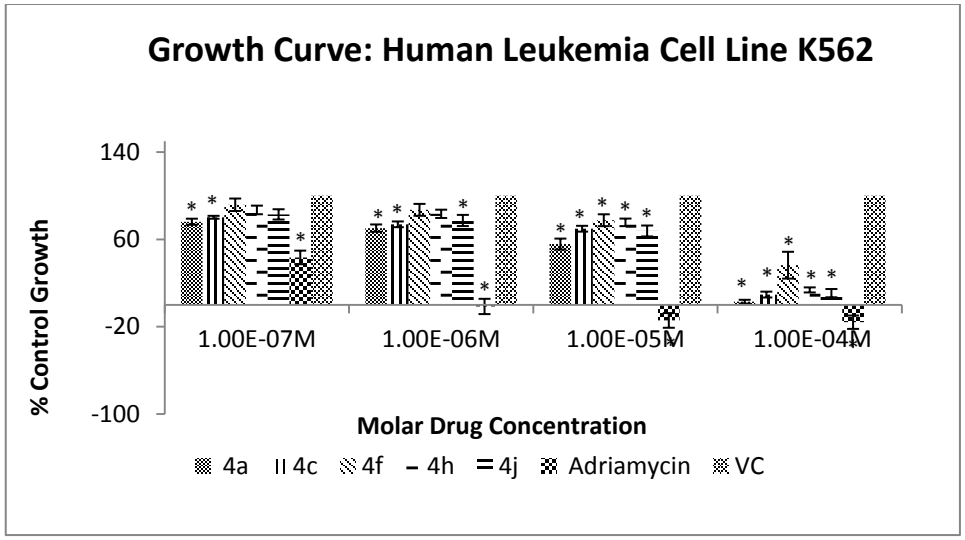


Figure 5. Effect of different chalcone derivatives on the percentage growth inhibition of the K562 cell line[Values are Mean ± SEM of 3 replicates each. *P<0.05, VC = Vehicle Control (DMSO)]

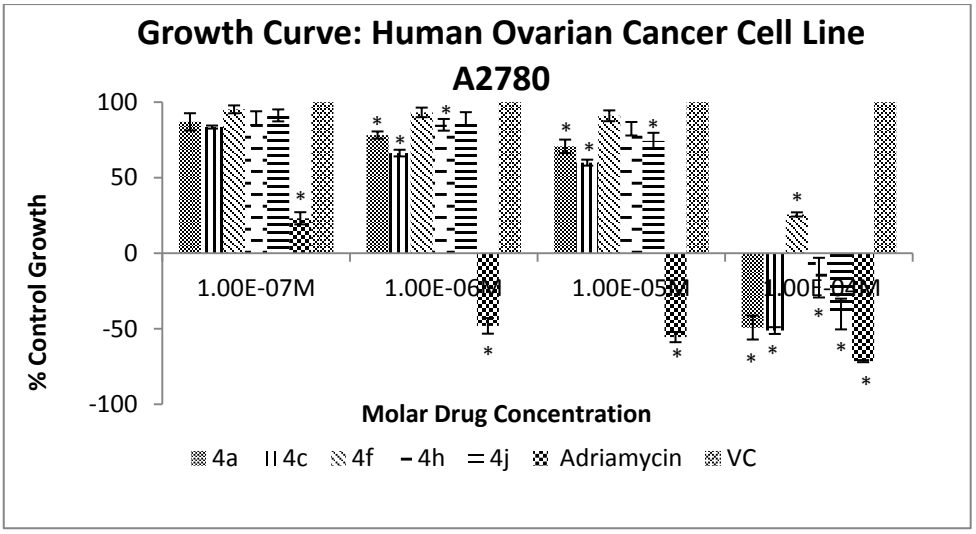


Figure 6. Effect of different chalcone derivatives on the percentage growth inhibition of the A2780 cell line[Values are Mean ± SEM of 3 replicates each. *P<0.05, VC = Vehicle Control (DMSO)]

Table 1. Comparison of the GI_{50} values (in μM) of selected chalcone derivatives on different cell lines

Compound	In vitro cytotoxicity GI_{50} (μM)				
	U373MG	MCF-7	HepG2	K562	A2780
4a	34.8	26.9	39.3	30.6	26.2
4c	33.9	31.9	38.7	41.0	20.5
4f	45.3	50.8	>100	73.5	66.4
4h	82.8	52.2	90.0	49.7	38.4
4j	35.6	38.1	41.1	42.8	31.5
Adriamycin	<0.1	<0.1	<0.1	<0.1	<0.1

Table 2. Comparison of the TGI values (in μM) of selected chalcone derivatives on different cell lines

Compound	In vitro cytotoxicity TGI (μM)				
	U373MG	MCF-7	HepG2	K562	A2780
4a	67.5	72.0	74.2	>100	63.9
4c	70.5	77.6	73.0	>100	60.2
4f	87.2	>100	>100	>100	>100
4h	>100	>100	>100	>100	85.9
4j	68.3	87.1	77.8	>100	69.9
Adriamycin	29.0	16.5	49.1	38.7	46.7

The TGI values represent a growth inhibition of the cell lines, where the cells are not growing as well as the control or are not killed or inhibited as well as incubation with the standard drug. The TGI values remain one of the factors for deciding the clinical efficacy of the tested compounds. The TGI values for the glioblastoma cell line ranged from 67-100 μM with the unsubstituted chalcone **4a** having the least value (Table 2). For the breast cancer cell line, the values ranged from 72-100 μM while the pancreatic cancer cell line exhibited a TGI value close to 75 μM. The human leukemia cell line was found to be insensitive with respect to the growth inhibiting concentration, with a TGI of 100 μM. The TGI values for the ovarian cancer cell lines ranged from 60-100 μM. It is to be noted that with the exception of the leukemia cell line, all the other cell lines exhibited TGI values that correlate well with the GI_{50} values.

3.2. Chemistry and Cytotoxic Activity

Although tubulin binding ability has been the most thoroughly investigated mode of action of chalcones, a number of other diverse mechanisms have been proposed that may explain their potent cytotoxic activity in diverse cell lines. Some of these include TRAIL-mediated as well as hypoxia-induced apoptosis, mitochondrial membrane disruption, inhibition of cell signal transduction as well as NF-κB (Nuclear Factor kappa-light chain-enhancer of activated B cells) pathways, and cell cycle arrest[27].

The general trend in the correlation of cytotoxicity with different A-ring substituents pointed to a net decrease in biological activity. Typically, the parent chalcone 4a was found to have a higher cytotoxic activity in relation to the GI_{50} values compared to the substituted derivatives. This may point to the pharmacological significance of an intact A-ring, but other studies[28] have reported higher cytotoxic activity of different A-ring substitutions compared to the unsubstituted compound. This leads us to the reasonable assessment that it is not the substitutions per se but the regiochemistry of substitution that has a profound impact on the biological activity. Another interesting observation that has been evident all through the study is the characteristic biphasic response (growth hormesis) of this class of compounds that has previously been reported[26, 29-31]. Many hypotheses have been propounded to shed light on this phenomenon. First, the proliferation control mechanisms in the cancer cell might respond to the low growth inhibiting concentration of chalcone by inducing regulatory corrections which might be disabled at higher concentrations leading to apoptosis[32]. Second, it is known that chalcones and other flavonoid moieties have a dynamic equilibrium between the anti-oxidant and pro-oxidant properties that is concentration

dependent. For the same reason, it can be concluded that a higher cellular chalcone concentration might contribute to oxidative reactions leading to build up of reactive oxygen species (ROS), which in turn induce cytotoxic response in cell cultures. However at lower concentrations, chalcones might act as anti-oxidants and protect the cells from oxidative damage[32]. Finally, the compounds might act as cell cycle specific modulators depending on the cellular concentrations and target the S phase at lower and G_1 phase at higher concentrations[29, 33]. The pro-oxidant activity of the flavonoid family of natural products including their biosynthetic precursor compounds in the form of chalcones has been studied in detail. Strikingly, one of the major mechanisms of cytotoxicity of these moieties at higher concentrations has been attributed to their pro-oxidant action[34, 35]. Furthermore, the catalyzing activity of endogenous peroxidases naturally present in cell cultures at higher concentrations may also trigger high ROS levels leading to oxidative stress, mitochondrial dysfunction and cell death[36, 37]. The work reported herein attempts to establish structure function correlations with regard to cytotoxicity and as such, a mechanistic investigation of these derivatives is out of scope of this study. However, given our observation of biphasic response in all the tested analogs coupled with the literature precedence, there is significant clarity with regard to the existence of a pro-oxidant machinery effecting cytotoxicity in cancer cells. Therefore, it can be reasonably hypothesized that the cytotoxic activity of these compounds is likely to arise at least in part due to their pro-oxidant activity.

From among the analogs evaluated, 4c bearing the 2'-methyl substituent was found to have comparable activity to the parent 4a. In this study, the 3'-nitro as well as the 4'-methoxy (4f and 4h respectively) derivatives were the worst affected in terms of their cytotoxic activity due to the substitutions on A-ring. Significantly, our results corroborate previous findings relating to the suppression of the cytotoxic effects due to the 4'-methoxy substitution on MCF-7 cell line[38]. The higher cytotoxicity of the unsubstituted chalcone compared to the different derivatives with substitutions on the A-ring alone as well as on both the rings has also been reported earlier. In another study, it was observed that the cytotoxicity of the 4'-methoxy derivative was drastically inhibited when chlorine was introduced at the *ortho* position of the A-ring, while there was a significant increase in the activity when a methyl group was introduced at the same position. The activity was again found to significantly increase by placing a hydroxy substituent at the *ortho* position in the A-ring[38]. These data clearly indicate that it is not only the nature and position of the substituent(s) on any particular ring, but also the interplay of different substitutions on other rings that can affect the overall biological activity of any compound[38]. Therefore, derivatives that are not significantly active in the current study may have greatly altered activity when combined with other substituents possessing characteristic electronic and steric properties.

4. Conclusions

In summary, we rationally designed and readily synthesized a set of monosubstituted chalcone derivatives and evaluated them for potential cytotoxic activity against five human cancer cell lines. Although all the compounds under investigation exhibited lesser GI_{50} values than adriamycin, some interesting observations could be made from the results. The unsubstituted parent chalcone **4a** was found to show higher cytotoxic activity than most of the substituted derivatives. In general, the substitutions at 2'- (*ortho*-), 3'- (*meta*-) or 4'- (*para*-) positions lead to a suppression of the cytotoxic activity. Among the substituted congeners investigated, the 2'-methyl derivative **4c** was found to exhibit the most potent cytotoxic activity. This study has thus provided valuable preliminary information on the effects of various substituents on the cytotoxic profile of chalcones. This in turn may act as an impetus for the design of more potent chalcone derivatives as anticancer leads. Further studies with larger libraries of compounds screened against diverse cell lines are needed to establish a strong and reliable antiproliferative SAR profile for these molecules.

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