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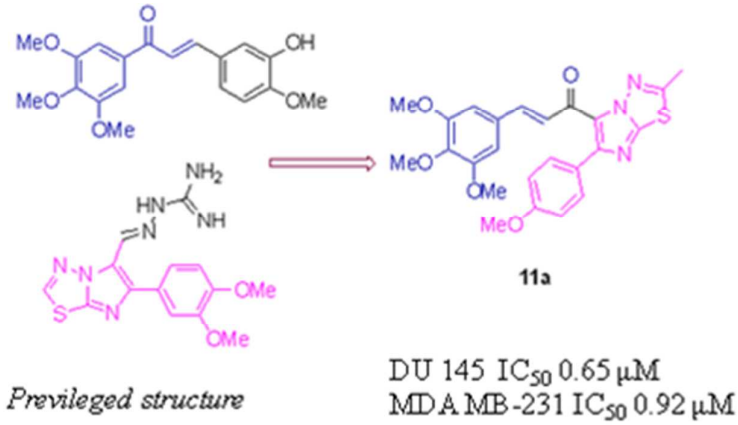
Graphical abstract

A library of imidazothiadiazole-chalcone conjugates were synthesised and investigated for their cytotoxic activity against various human cancer cell lines. Some of the tested compounds like **7a**, **7b**, **11a** and **11b** exhibited promising anticancer activity.



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ARTICLE

Synthesis of imidazo[2,1-b][1,3,4]thiadiazole-chalcones as apoptosis inducing anticancer agents

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Abstract: A series of new imidazo[2,1-*b*][1,3,4]thiadiazole-chalcones were synthesized by the Claisen-Schmidt condensation and evaluated for their cytotoxic activity against various human cancer cell lines. These compounds showed moderate to appreciable antiproliferative activities. Interestingly, compounds like **11a** and **11b** exhibited significant cytotoxic activity with IC_{50} values ranging from 0.65 to 2.25 μM in certain cancer cell lines. The structure activity relationship (SAR) studies reveal that 3,4,5-trimethoxy group containing compounds showed superior cytotoxic activity against selected cancer cell lines compared to other chalcones. These compounds showed G_0/G_1 phase arrest, apart from activation of caspase-3 and 8 in DU-145 cells. The growth inhibitory effect of these compounds was associated with a decrease in cell cycle regulatory protein cyclin D1 and increase in cyclin dependent kinase inhibitors like Cip1/p21 and Kip1/p27.

Introduction

Chalcones, represent an important class of natural products, which are intermediate precursors of all flavonoid based compounds.[1-4] Chalcones are interesting simple molecules due to their varied biological activities including anti-inflammatory,[5] anti-malarial,[6,7] antituberculosis,[8] anti–HIV,[9] antiproliferative,[10-13] and inhibition of several enzymes like aromatase, topoisomerase, certain protein-tyrosine kinases like cyclin-dependent kinases.[14] They display significant biological activities due to their potential interactions with different proteins related to cell proliferation and apoptosis. Recent results show that chalcone derivatives can induce apoptosis in cancer cells, [15-17] and most of them contain either hydroxy or methoxy groups in both the rings (A and B). The structure activity relationship (SAR) studies of trimethoxy chalcones (TMC) (**II**) reveal that the 3,4,5-trimethoxyphenyl group in ring A is thought to be essential for the anticancer activity. Some of the recent studies towards the improvement of their cytotoxicity and to the impact of different substituents in the aryl ring were studied extensively. Moreover, chalcone derivatives in which the ring B is replaced by a heterocyclic ring have been systematically investigated.[18,19]

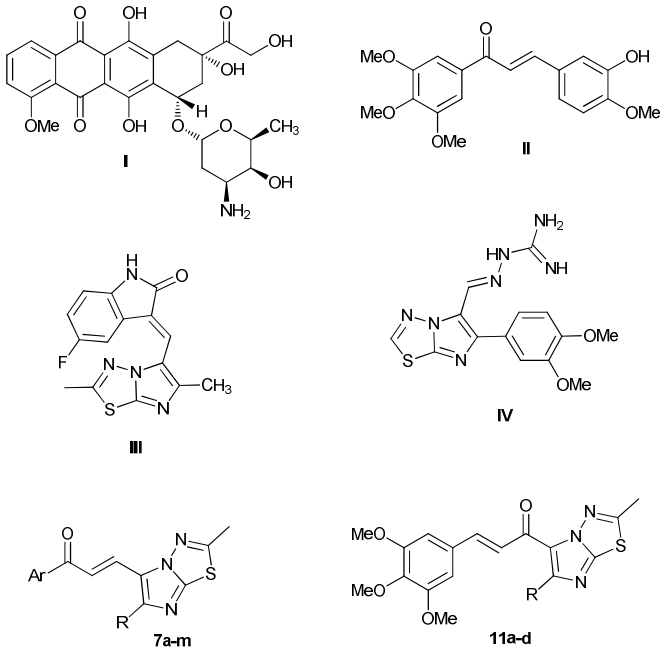


Figure 1. Structures of anticancer agent Doxorubicin (**I**), Trimethoxychalcone (TMC) (**II**), Imidazothiadiazole-oxindole (**III**), Imidazothiazole guanylhydrazone derivative (**IV**) and Imidazothiadiazole-chalcones (**7a-m** and **11a-d**).

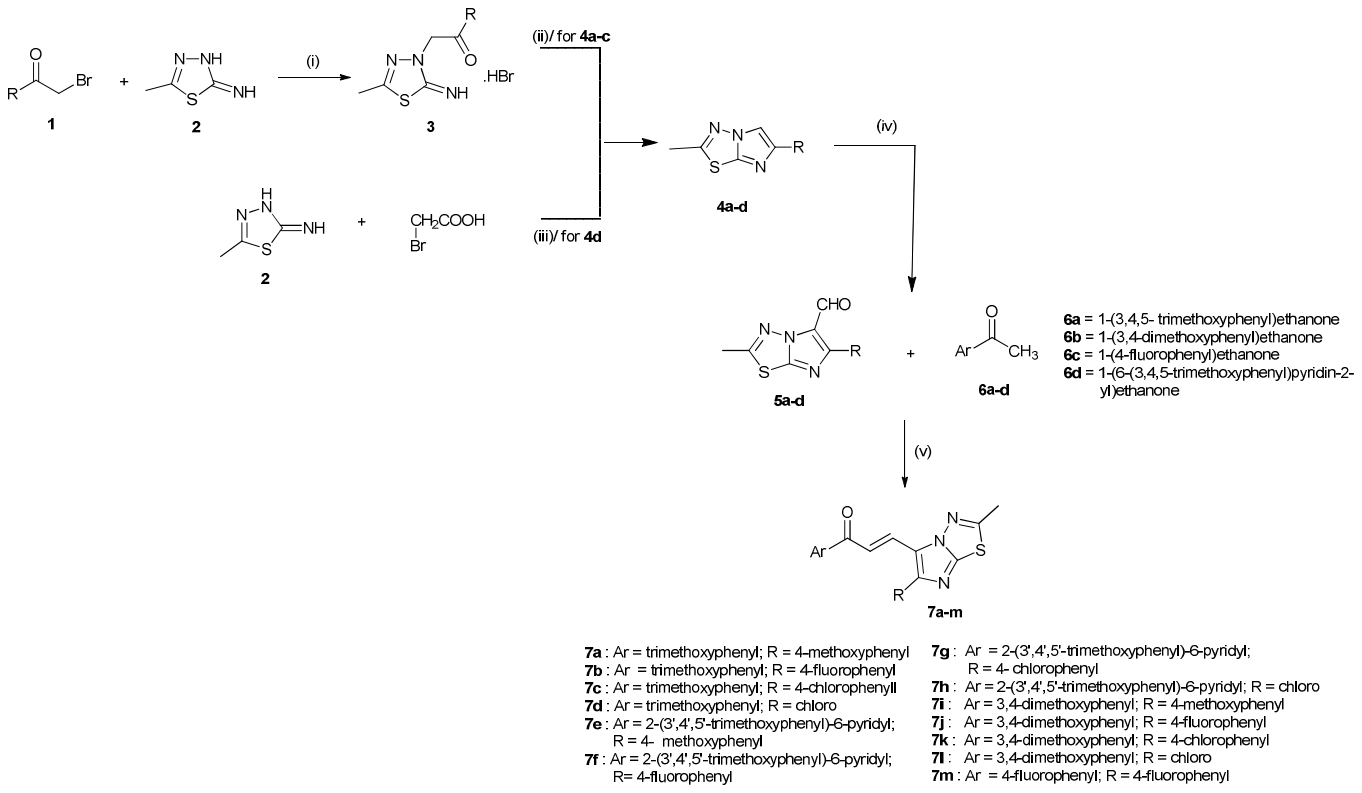
Imidazo[2,1-*b*]thiadiazoles (**III**, **IV**) (Figure 1) are familiar compounds and their derivatives are known to exhibit a wide spectrum of biological activities like antimicrobial,[20] antibacterial,[21] antitubercular[22] and anticancer activity.[23] Several representative chalcones and imidazothiadiazole derivatives are shown in Figure 1. By keeping these structural aspects of chalcones and imidazothiadiazoles, we have designed and synthesized a new class of imidazothiadiazole linked chalcones (**7a-m**, **11a-d**) and evaluated their anticancer potential. Some of these conjugates showed excellent antiproliferative effects in certain cancer cell lines. Moreover, to understand the possible mechanisms involved in mediating the antiproliferative effect, we have studied the cell cycle alterations and apoptotic markers in DU-145 prostate cancer cells.

Results and discussion

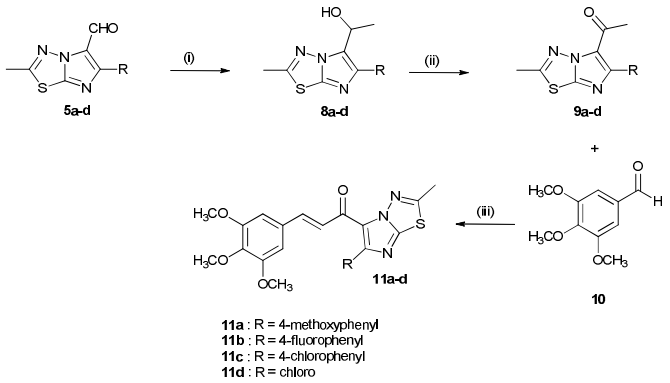
Chemistry

The imidazothiadiazole-chalcones (**7a-m**) were prepared by the Claisen-Schmidt condensation[24] of appropriate substituted acetophenones (**6a-d**) upon treatment with imidazo[2,1-*b*]thiadiazole aldehydes (**5a-d**) in the presence of NaOH (10%) as shown in Scheme 1. The imidazo[2,1-*b*]thiadiazole aldehydes[25] were obtained by means of the Vilsmeier reaction on the corresponding imidazo[2,1-*b*]thiadiazole (**4a-d**), wherein **4a-c** were prepared from the appropriate 5-methyl-1,3,4-thiadiazol-2-amine (**2**) and bromoketones (**1**), however, **4d** is prepared from **2** and 2-bromoacetic acid as illustrated in Scheme 1.

Whereas, imidazothiodiazole-chalcone derivatives (**11a-d**) were obtained by the Claisen-Schmidt condensation of appropriate substituted imidazothiadiazole ethanones (**9a-d**) upon treatment with 3,4,5-trimethoxybenzaldehyde (**10**) in the presence of NaOH (10%) as shown in Scheme 2. Imidazothiadiazole ethanones (**9a-d**) were prepared by Grignard reaction of the corresponding imidazo[2,1-*b*]thiodiazole aldehydes (**5a-d**) and followed by oxidation with IBX in DMSO.



Scheme 1. (i) acetone, reflux, 6-8 h; (ii) 2N HCl, reflux, 1 h, 85-95%; (iii) C_2H_5OH, $POCl_3$, reflux, 7-9 h, 20% NH_3, 70-80%; (iv) $POCl_3$, DMF, reflux for 3 h, 70-80%; (v) 10% aq. NaOH, 12 h, rt, 75-85%.



Scheme 2. (i) CH3MgBr, THF, 5 ^{o}C 6-8 h; (ii) IBX, DMSO, 0 ^{o}C, 2 h, 55-65%; (iii) 10 % aq. NaOH, 12 h, rt, 75-85%.

Evaluation of biological activity

Anticancer activity

The imidazothiadiazole-chalcones (**7a-m** and **11a-d**) were evaluated for their cytotoxic activity against various human cancer cell lines of prostate, breast, and lung, by using MTT assay method.[26, 27] All the chalcone derivatives showed moderate to good cytotoxic activity with IC_{50} values ranging from 0.65 to 18.64 μM. Interestingly, compounds **11a** and **11b** showed significant growth inhibitory/cytotoxic activity against a number of cancer cell lines tested in this study (Table 1). The structure activity relationship (SAR) studies reveal that 3,4,5-trimethoxy group containing compounds (**7a**, **7b**, **7c**, **11a**, and **11b**) showed superior cytotoxic activity against selected cancer cell lines compared to the other chalcone derivatives. Compounds having a pyridine moiety (**7e**, **7f**, **7g** and **7h**) showed less activity with comparison to other chalcones. Moreover, compounds **7d**, **7h**, **7l** and **11d** exhibited a reduced amount of activity, due to substituted phenyl ring replaced by simple chlorine atom. Similarly, compounds **7c** and **11c** contains chlorine (Cl) substitution on phenyl ring, showed less cytotoxic activity than **7a-b** and **11a-b** (OCH_3 and F substituted phenyl ring). In addition, compound **7m** (has two fluorine atoms on phenyl rings) also showed slightest activity. It is observed that the potent compounds **11a** and **11b** possess a *trans* double bond beside the aryl group however in compounds **7a** and **7b,** the double bond is attached to the imidazothiadiazole ring. The cytotoxic activity of these imidazothiadiazole-chalcone derivatives is comparable to doxorubicin (**I**), a well known chemotherapeutic drug and more potent than trimethoxy chalcone (**II**). Moreover, imidazothiadiazole-oxindole derivative showed moderate activity.[23] In addition, intermediates **5a-b** and **9a-b** exhibited poor cytotoxic activities than **7a-m** and **11a-d**. Interestingly, compounds **11a** and **11b** showed better antiproliferative activity with IC_{50} values ranging from 0.65 to 2.25 μM than doxorubicin (2.45 to 3.41 μM) against tested cell lines and the results are summarized in Table 1.

Table 1. IC_{50} values (in μM)[a] for compounds **7a-m** and **11a-d** in selected human cancer cell lines

Compound	IC_{50} values(μM)[a]			
	DU 145[b]	MDA MB-231[c]	MCF-7[c]	A549[d]
5a	47.23	-	39.43	-
5b	59.46	-	38.69	-
9a	> 100		98.07	-
9b	> 100		> 100	-
7a	**3.56**	**4.32**	**2.78**	**4.24**
7b	**3.25**	**4.65**	**5.82**	**6.68**
7c	5.89	2.46	4.24	6.68
7d	15.38	11.85	5.91	9.54
7e	10.25	5.32	9.67	14.62
7f	15.32	15.96	12.65	14.65
7g	13.58	11.32	9.89	17.68
7h	10.32	11.35	8.95	14.87
7i	4.24	6.36	4.95	5.34
7j	4.89	4.36	5.78	5.92
7k	5.32	6.75	6.92	5.65
7l	15.39	9.25	12.32	19.32
7m	17.67	16.02	18.64	17.68
11a	**0.65**	**0.92**	**1.34**	**2.25**
11b	**0.89**	**1.32**	**1.27**	**1.90**
11c	**2.91**	**3.39**	**2.64**	**4.28**
11d	15.39	12.34	19.34	24.36
Doxo (I)	2.45	3.41	3.12	2.10
TMC (II)	4.07	-	4.67	2.95

[a] Values are mean of three independent experimental determinations, [b] prostate cancer , [c] breast cancer, [d] lung cancer cell lines.

Effect on cell cycle arrest

To examine the mechanism underlying the antiproliferative effect of these imidazothiadiazole-chalcones, we analyzed the cell cycle alterations phase distribution and the DNA content of the cell by treating DU-145 cells with them at 3 µM concentration for 24 h by flow cytometry. The effect of these compounds (**11a** and **11b**) and doxorubicin (employed as a standard) on cell cycle events was analyzed. Treatment of DU-145 cells with compounds **11a** and **11b** caused an accumulation of 83 % and 81 % cells in G_0/G_1 phase respectively as compared to 65 % in untreated cells. On the other hand, doxorubicin has shown 92 % cells in G_0/G_1 phase (Figure 2 and Table 2).

Table 2 cell cycle distribution of compounds **11a, 11b** and doxorubicin in DU145 cells

	G0/G1	S	G2/M
Control	65	10	25
11a	83	8	9
11b	81	11	8
Dox(**I**)	92	5	3

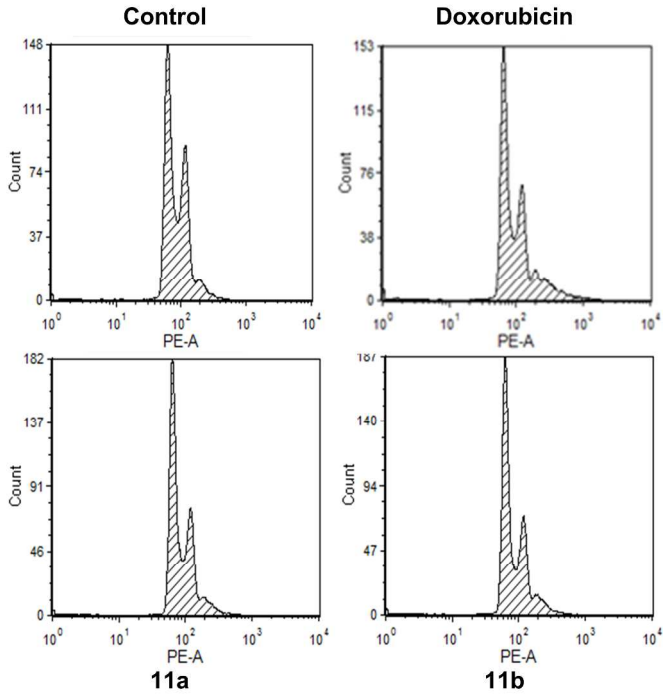


Figure 2. Effect of compounds **11a** and **11b** on cell cycle arrest. DU-145 cells were treated with compounds **11a, 11b** and doxorubicin (3 µM) for 24 h. Untreated cells and DMSO-treated cells served as controls. Cell-cycle analysis was performed with propidium iodide as indicated in the Experimental Section. The cell-cycle phase distribution was determined by FACS and the percentage of cells in each phase was analyzed by FCS express 4 plus.

Effect on chromatin condensation

Apoptosis is an important process of cell death of undesirable cells during development or homeostasis in multicellular organisms and during apoptosis, chromatin condensation takes place.[28] To see whether imidazothiadiazole-chalcone conjugates (**11a** and **11b**) induced cytotoxicity occurs through apoptosis, DU-145 prostate cancer cells were treated with 3 µM concentration of these compounds for a period of 24 h. Hoechst 33258 staining was used to visualize nuclear condensation. It was found that both these compounds caused a significant nuclear condensation as shown in Figure 3. Doxorubicin, a known inducer of cell death by apoptosis was used as a positive control at the same concentration.

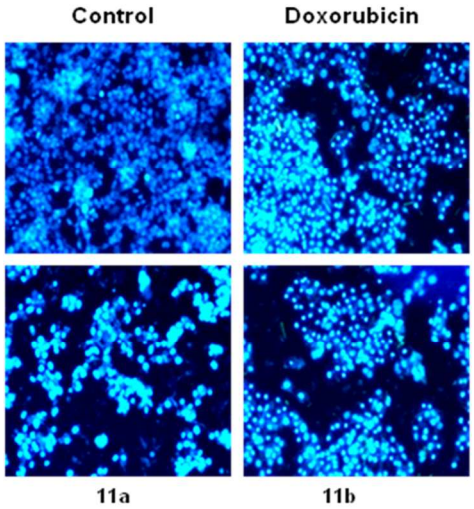


Figure 3. Imidazothiadiazole-chalcone compounds cause apoptosis in DU-145 cells. Cells were treated with **11a**, **11b** and doxorubicin at 3 µM concentration for 24 h and washed with PBS, incubated with Hoechst-33258 stain (4 mg/mL) for 20 min to measure chromatin condensation. Images were photographed using fluorescenece microscopy equipped with DAPI filter.

Caspases-3 and 8 activation

We measured the activation of caspase-3 and caspase 8 for confirmation of the chromatin condensation results.[29-31] DU-145 cells treated with compounds (**11a** and **11b**) along with doxorubicin at 3 µM concentration for 24 h. It was found that both the compounds significantly activated caspase-3 and 8, as in case of doxorubicin (**I**). These results reveal that imidazothiadiazole-chalcone conjugates (**11a** and **11b**) induce DU-145 cell death by apoptosis (Figure 4).

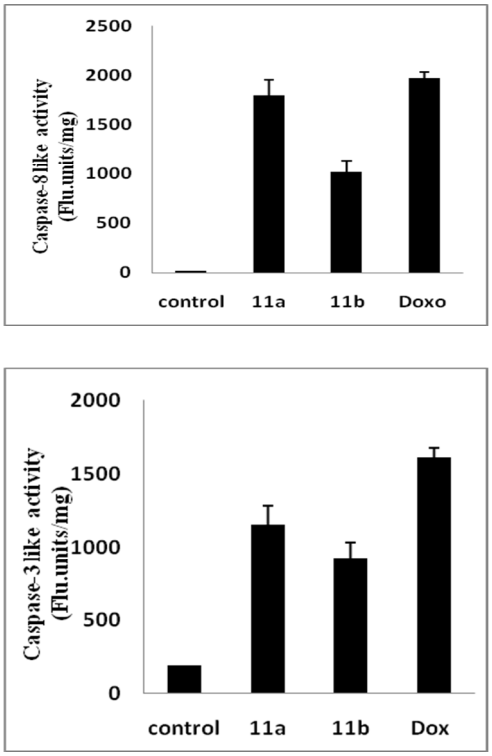


Figure 4. Effect of compounds **11a** and **11b** on caspase-3 and 8 activity as a measure of apoptosis. DU-145 cells were treated with compounds **11a**, **11b** and doxorubicin at 3 μM concentration for 24 h and cell lysates were incubated with the fluorogenic caspase-3 and 8 substrates DEVD-AFC and IETD-AFC respectively for 1 h at 37 ^{o}C and released AFC fluorescence was measured in a multimode reader using an excitation /emission of 400/500 nm.

Effect of imidazothiadiazole-chalcone derivatives on cell-cycle regulatory proteins

As cell cycle progression from G_0 through G_1 phase involves activation of the cell cycle regulatory proteins like cyclin D1 and E,[32-36] therefore we invesigated the effect of compounds **11a** and **11b** on the cell cycle proteins. It was observed that these conjugates along with doxorubicin down-regulated cyclin D1 and cyclin dependent kinase (CDK) 4 in cells treated with 3 μM concentration of the respective compounds for a period of 24 h. Cyclin D1 levels decreased by both **11a** and **11b**, however, the levels of cyclin E were regulated down with only **11a**. In tune with the altered cyclin D1 and E expression profiles, the levels of p21 and p27 cyclin dependent kinase inhibitors were significantly increased (Figure 5).

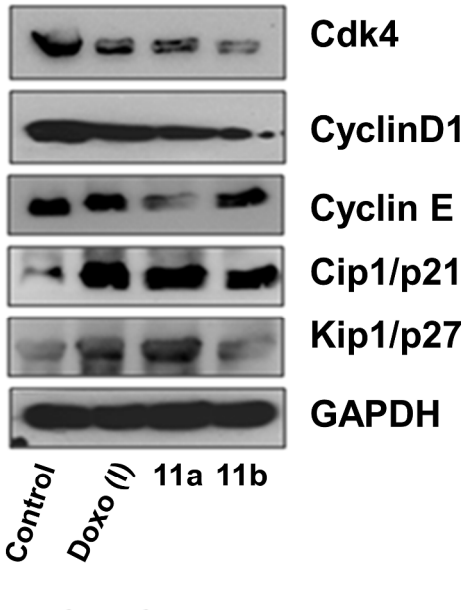


Figure 5. Effect of compounds **11a** and **11b** on cell cycle protein alterations. DU145 cells were treated with **11a**, **11b** and doxorubicin (**I**) at 3 μM concentration for 24 h. Cell cycle proteins (CDK4, CyclinD1, Cyclin E, p21and p27) and GAPDH were measured by western blot analysis as described in the experimental section.

Conclusion

In conclusion, a new class of imidazothiadiazole-chalcones were synthesized and evaluated for their cytotoxic activities against four representative human cancer cell lines. All the modified chalcone derivatives showed moderate to good cytotoxic activity against the cancer cell lines tested in this study. Among them compounds **11a** and **11b** exhibited significant anti-cancer potency with IC_{50} values ranging from 0.65 to 2.25 μM. The results revealed that compounds **11a** and **11b** caused cell cycle arrest with majority of population of cells accumulated in the sub-G1 phase suggesting that these compounds have the capability to induce cell death by apoptosis. Further, the role of cell cycle regulatory proteins provide the mechanism for the cell growth inhibitory properties of these compounds demonstrating the down-regulation of CDK4 and cyclin D1 proteins because of the increase in G1/S checkpoint-associated tumor suppressor proteins p21 and p27. Overall, the present study shows that these new heterocyclic chalcones exhibit promising cytotoxic activity and has the potential to the taken up as new leads for the treatment of cancer.

Acknowledgements

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Captions

Figure 1. Structures of anticancer agent Doxorubicin (**I**), Trimethoxychalcone (TMC) (**II**), Imidazothiadiazole-oxindole (**III**), Imidazothiazole guanylhydrazone derivative (**IV**) and Imidazothiadiazole-chalcones (**7a-m** and **11a-d**).

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Table 1. IC_{50} values (in μM)[a] for compounds **7a-m** and **11a-d** in selected human cancer cell lines

[a] Values are mean of three independent experimental determinations, [b] prostate cancer , [c] breast cancer, [d] lung cancer cell lines.

Table 2 cell cycle distribution of compounds **11a, 11b** and doxorubicin in DU145 cells

Figure 2. Effect of compounds **11a** and **11b** on cell cycle arrest. DU-145 cells were treated with compounds **11a, 11b** and doxorubicin (3 μM) for 24 h. Untreated cells and DMSO-treated cells served as controls. Cell-cycle analysis was performed with propidium iodide as indicated in the Experimental Section. The cell-cycle phase distribution was determined by FACS and the percentage of cells in each phase was analyzed by FCS express 4 plus.

Figure 3. Imidazothiadiazole-chalcone compounds cause apoptosis in DU-145 cells. Cells were treated with **11a**, **11b** and doxorubicin at 3 μM concentration for 24 h and washed with PBS, incubated with Hoechst-33258 stain (4 mg/mL) for 20 min to measure chromatin condensation. Images were photographed using fluorescenece microscopy equipped with DAPI filter.

Figure 4. Effect of compounds **11a** and **11b** on caspase-3 and 8 activity as a measure of apoptosis. DU-145 cells were treated with compounds **11a**, **11b** and doxorubicin at 3 μM concentration for 24 h and cell lysates were incubated with the fluorogenic caspase-3 and 8 substrates DEVD-AFC and IETD-AFC respectively for 1 h at 37 ^{o}C and released AFC fluorescence was measured in a multimode reader using an excitation /emission of 400/500 nm.

Figure 5. Effect of compounds **11a** and **11b** on cell cycle protein alterations. DU145 cells were treated with **11a**, **11b** and doxorubicin (**I**) at 3 μM concentration for 24 h. Cell cycle proteins (CDK4, CyclinD1, Cyclin E, p21and p27) and GAPDH were measured by western blot analysis as described in the experimental section.