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Synthesis and biological evaluation of cinnamido linked benzophenone hybrids as tubulin polymerization inhibitors and apoptosis inducing agents

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ABSTRACT

A new class of hybrid molecules containing cinnamide subunit linked to benzophenone as inhibitors of tubulin polymerization were synthesized and evaluated for their anticancer potential. These hybrids exhibit anticancer activity with IC_{50} values ranging from 0.06 to 16.3 μM. Compounds **4f** and **4g** possessing fluoro and trifluoromethyl on the cinnamido subunit showed significant cytotoxic activity with IC_{50} values 0.06 and 0.09 μM against HeLa cell line, respectively. These compounds showed cell cycle arrest at G2/M phase of the cell cycle and inhibited tubulin polymerization followed by activation of caspase-3 activity and apoptotic cell death. Further in vitro tubulin polymerization assay showed that the level of tubulin inhibition was comparable to that of **2a** for the compounds **4f** and **4g**. Moreover, Hoechst 33258 staining and DNA fragmentation assay suggested that these compounds induce cell death by apoptosis. Overall, the current study demonstrates that the synthesis of benzophenone linked cinnamide subunit conjugates as promising anticancer agents with G2/M arrest and apoptotic-inducing ability via targeting tubulin.

Cancer, a disease of uncontrolled mitosis that represents approximately 200 different diseases, is now one of the most frequent causes of death and is the second leading cause of death. It is estimated that new cancer diagnoses will double by 2020 and nearly triple by 2030.[1] Despite the fact, that many anticancer drugs are in clinical use through different mechanism of action, their action is not specific. Moreover the drugs used in chemotherapy are limited by drug resistance and thereby there is an urgency to develop molecules that could selectively deliver their action at the tumor site.

Among the current cellular targets in eukaryotes, alongside DNA, tubulin-microtubule dynamics are critical for mitotic spindle formation during cell division and are a promising target for anticancer agents.[2–4] These microtubules, comprising of α and β-tubulin heterodimers, are always in a state of equilibrium with the latter with a certain amount of composition. Ligands perturb these dynamics by binding to either tubulin or microtubule, leading to the inhibition of microtubule polymerization or depolymerization towards forming abnormal mitotic spindle and ultimately resulting in mitotic arrest.[5] Well known examples include paclitaxel, vinblastine, epothilone, dolastatin and colchicine. In addition some of these ligands target tumor endothelial cells which results in a rapid occlusion of tumor vasculature, leading to vascular shutdown.

Belonging to this class of molecules, phenstatin (**1a**, Fig. 1), a benzophenone motif reported by Pettit and co-workers, showed potent anticancer and antimitotic activities.[6,7] A phosphate prodrug of phenstatin (**1b**) was also reported. Later, Hsieh and co-workers, introduced an amino group at C-3 position of the B-ring resulted in **2a**, that exerts potent tubulin polymerization inhibition and shows promising cytotoxic activity against selected human cancer cell lines.[8] Very recently Lee et al. also demonstrated a new class of benzophenone series with insertion of small heterocyclic groups in the B-ring.[9] In addition several others reported analogues of phenstatin[10] and aryl–heteroaryl ketones (indole, quinoline, carbazole, thiophenes) as potential tubulin inhibitors.[11–14]

At the same time we came across several antitumor molecules possessing cinnamido scaffold that exhibit anticancer activity by different mechanism of action.[15] Hergenrother and coworkers in 2010 reported simple phenyl cinnamides acting as antimitotic agents by disruption of microtubule dynamics that leads to cell

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Table 1
Structural representation of the synthesized compounds and their codes

Compd	R^1	R^2	R^3	Compd	R^1	R^2	R^3
4a	OMe	OMe	OMe	**4l**	H	NO_2	H
4b	OMe	OMe	H	**5a**	OMe	OMe	OMe
4c	H	OMe	H	**5b**	OMe	OMe	H
4d	OMe	H	H	**5c**	H	OMe	H
4e		H	H	**5d**	OMe	H	H
4f	H	F	H	**5e**	NH_2	OMe	H
4g	H	CF_3	H	**5f**		H	H
4h	OMe	F	H	**5g**	H	F	H
4i	OCF_3	H	H	**5h**	H	CF_3	H
4j	F	F	H	**5i**	F	F	F
4k	H	NH_2	H				

cycle arrest in G2/M phase; among which **3** displayed promising activity.[16] In continuation of our efforts towards the design of new anticancer agents,[17–19] we considered it worthwhile to pursue further modifications on the benzophenone part by appending cinnamide subunit to the amine functionality situated at 3′-position on the B-ring of the benzophenone, that may allow better interactions at the receptor site and could affect the biological activity. This report describes the synthesis and evaluation of their anticancer activity apart from their effect on tubulin polymerization, cell cycle and apoptosis inducing ability of these hybrids.

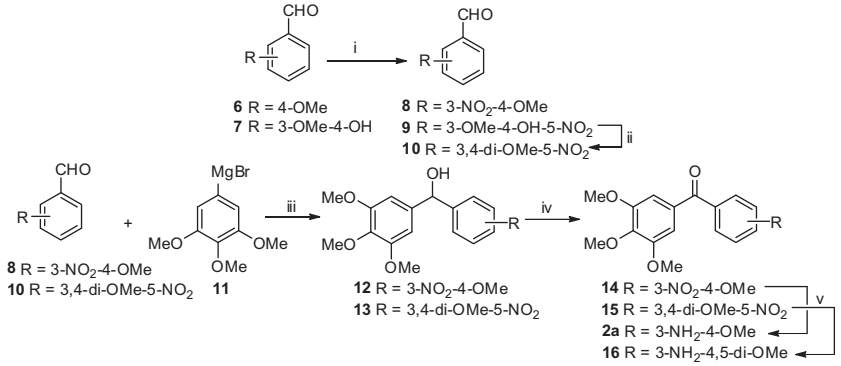
Results and discussions

Benzophenone–cinnamides **4a–l** and **5a–i** listed in Tables 1 were synthesized as depicted in Schemes 1 and 2. The required aldehydes **8** and **10** were prepared by nitration of **6** and **7** using 70% HNO_3 according to the reported literature procedures.[20] As presented in Scheme 1, benzhydroxy compounds **12** and **13** obtained from Grignard reaction between **11** and **8** or **10**, were oxidized using pyridinium dichromate to afford the nitro substituted benzophenone derivatives **14** and **15**. Reduction of nitro group of **14** and **15** was achieved using Fe-AcOH/EtOH under reflux conditions to provide the benzophenone amine precursors **2a** and **16**.[8]

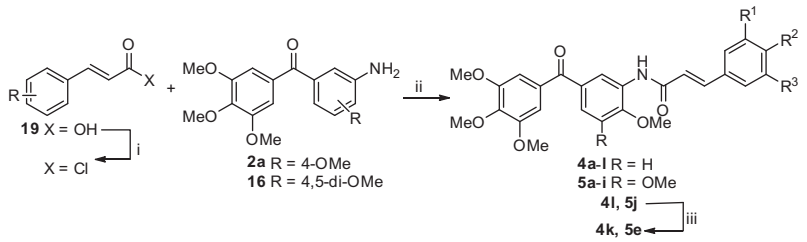
Table 2
Cytotoxicity (IC_{50} μM)[a] data of compounds **4a–l** and **5a–i**

Compd	HeLa[b]	ME-180[b]	DU-145[c]	PC-3[c]	COLO205[d]	HT-29[d]	B-16[e]
4a	1.5	2.1	6.3	3.9	5.2	3.1	1.2
4b	7.8	8.9	10.2	7.5	8.2	2.5	6.3
4c	10.7	11.8	12.8	11.1	10.9	5.01	8.7
4d	3.1	4.3	6.2	4.7	5.1	1.9	3.1
4e	14.0	15.3	15.8	13.1	13.4	7.4	12.2
4f	0.06	0.3	3.9	3.2	3.4	1.8	0.4
4g	0.09	0.8	4.3	3.5	2.6	1.6	0.1
4h	13.6	15.5	12.1	14.1	11.8	7.7	16.3
4i	7.9	9.2	10.2	7.7	7.9	5.2	6.3
4j	8.1	8.9	10.2	8.3	6.02	4.2	6.5
4k	14.7	16.0	13.5	15.9	10.3	5.6	7.9
4l	8.5	9.3	15.6	10.4	11.4	5.1	6.4
5a	2.5	3.9	6.6	4.8	5.3	3.1	2.5
5b	2.7	6.7	7.8	5.4	5.7	2.3	1.3
5c	11.2	12.5	10.4	6.3	4.8	3.8	9.4
5d	6.9	8.6	12.5	13.3	10.7	8.7	6.7
5e	15.8	13.9	12.7	11.1	10.1	7.7	12.7
5f	7.3	8.4	13.5	10.1	11.2	8.1	5.1
5g	0.9	1.0	6.7	4.1	3.1	1.8	0.9
5h	3.0	3.6	5.1	4.2	2.5	1.9	3.3
5i	5.0	7.1	7.9	7.4	4.3	3.3	5.2
16	13.5	12.2	10.0	7.4	4.4	3.8	12.5
2a	0.06	0.08	2.1	1.1	0.2	0.05	0.03
3	12.3	17.3	3.2	2.1	4.2	2.4	8.5

[a] Compound concentration required to inhibit cell proliferation by 50%.
[b] Cervical cancer cell lines.
[c] Prostate cancer cell lines.
[d] Colon carcinoma.
[e] Mouse melanoma carcinoma.



Scheme 1. Reagents and conditions: (i) 70% HNO_3, 8 h, rt for **6**; HNO_3, AcOH, 10 h, rt for **7**; (ii) Bu_4NBr, NaOH, Me_2SO_4, CH_2Cl_2/H_2O, 24 h, rt for **9**; (iii) THF, 0 °C for 10 min–rt, 1 h; (iv) PDC, dry CH_2Cl_2, 8 h; (v) Fe, AcOH–EtOH, 1–2 drops of HCl, reflux, 1 h.



Scheme 2. Reagents and conditions: (i) $(COCl)_2$, dry CH_2Cl_2, 0 °C, DMF (cat), 4 h; (ii) cinnamoyl chloride, Et_3N, THF, 0 °C 6 h; (iii) Zn, HCO_2NH_4, MeOH, rt, 6 h.

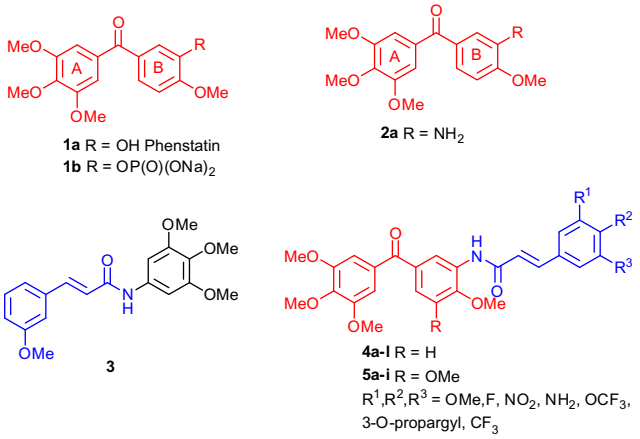


Figure 1. Tubulin polymerization inhibitors.

Finally the targeted hybrids **4** and **5** were obtained by the coupling of the substituted cinnamoyl chlorides to the amine intermediates **2a** or **16** in dry THF at 0 °C using triethyl amine (Et_3N) as base (Scheme 2). Cinnamoyl chlorides were obtained from the corresponding acids **19** using oxalyl chloride and catalytic amount of DMF. Some of the cinnamic acids were prepared according to the standard literature procedures; propargylation, Wittig reaction followed by saponification. Compound **4k** and **5e** were obtained by reduction of the corresponding nitro intermediates using zinc-ammoniumformate.

The synthesized compounds **4a–l** and **5a–i** were initially screened for their cytotoxic activity in selected human cancer cell lines of cervical, prostate, colorectal and mouse melanoma origin by using MTT assay.[21] All the compounds exhibit cytotoxicity with IC_{50} values ranging from 0.06 to 16.0 μM and the results are illustrated in Table 1.

Firstly the effect of the cinnamide moiety in compounds **4a–l**, possessing 4-OMe substitution on B-ring is examined. As shown in the Table 2, of the hybrids bearing methoxy substitution (**4a–c**); **4a**, possessing tri-OMe unit, exhibited good cytotoxicity with IC_{50} of 1.5, 2.1, 3.9, 3.1 and 1.2 μM against HeLa, ME-180, PC-3, HT-29 and B-16 cells; while activity was reduced for compound **4b** and **4c** (bearing 3,4-di-OMe and 4-OMe, respectively) in the cell lines tested. Interestingly **4d** (3-OMe substitution) exhibited cytotoxicity almost similar to **4a** with IC_{50} value of 1.96.2 μM range in all the tested cell lines. Whereas **4e**, replacing the methoxy group on **4d** with an *O*-propargyl substituent, resulted in reduced cytotoxicity. In this series **4f** and **4g**, having 4-F and 4-CF_3 substitution on the cinnamide phenyl ring, showed promising activity with IC_{50} of 0.06 and 0.09 μM against HeLa; 0.3 and 0.8 μM against ME-180 and 0.4 and 0.1 μM against B-16 cell lines, respectively, while with an IC_{50} range of 1.6–4.3 μM on the other cell lines (DU-145, PC-3 and COLO-205). **4f** and **4g** displayed same activity against HeLa as the corresponding parent core amine **2a** (IC_{50} of 0.06 μM) which is a positive control and showed far better activity than the cinnamide **3**. Introduction of methoxy or fluoro group at 3′-position to **4f** resulted in compounds **4h** and **4j** that showed decreased cytotoxicity and **4i**, possessing 3-OCF_3, exhibited moderate activity (IC_{50} of 5.2–10.2 μM). Whereas **4l** with 4-NO_2 substitution exhibited good activity (IC_{50} of 5.1–10.4 μM range) compared to **4k** (5.6–16.0 μM) that has a 4-NH_2 substituent.

We then evaluated the cytotoxicity of compounds **5a–i**, that has 3,4-di-OMe substitution on the B-ring of the benzophenone subunit apart from other substitutions in the cinnamido subunit. Compounds **5a** and **5b**, having tri-OMe and di-OMe substitution on the cinnamido ring, respectively, were found to display almost similar range of activity with IC_{50} 1.3–7.8 μM in the corresponding tested cell lines, unlike in case of **4a** and **4b** of the previous series. Compound **5c** with 4-OMe substitution showed reduced activity compared to **5b** (3,4-di-OMe) against HeLa, ME-180 and B-16 cell lines with IC_{50} of 11.2, 12.5 and 9.4 μM, while maintaining the same range of activity on the other cell lines. Whereas **5d** (3-OMe) showed increased cytotoxicity (IC_{50} of 6.9 and 8.6 μM) against HeLa and ME-180 compared to **5c** with reduced activity on PC-3, COLO-205 and HT-29 cell lines. Moreover **5e** (4-OMe-3-NH_2) also showed loss of activity on all the cell lines when compared to **5b**. Amongst the compounds of this series, **5g** bearing 4-F substitution showed significant IC_{50} value of 0.9, 1.0 and 0.9 μM against HeLa, ME-180 and B-16 cell lines. However **5h**, bearing 4-CF_3 substitution, showed reduced cytotoxic activity with

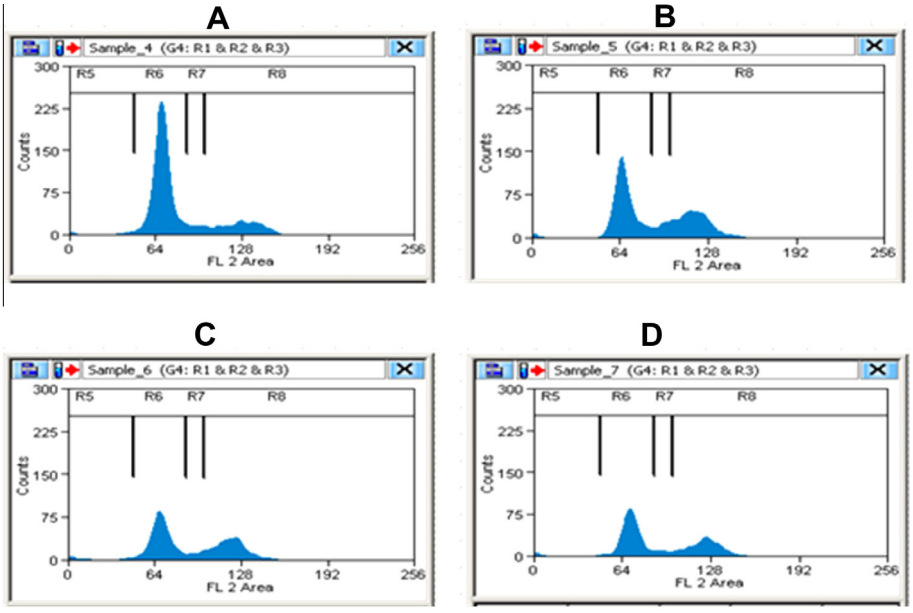


Figure 2. Effects of compounds A (control), B (**4f**), C (**4g**), and D (**2a**) on DNA content/cell following the treatment of HeLa cells at 0.5 μM for 24 h. Cell cycle distribution was analyzed by the standard propidium iodide procedure as described in Experimental section.

Table 3
Cell cycle distribution of HeLa cells (%) for **4f**, **4g** and **2a**

Compd	SUB-G1	G1 phase	S phase	G2/M
Control	3.07	71.21	4.62	21.10
4f	2.96	53.02	7.40	36.62
4g	5.66	49.59	5.92	38.82
2a	4.56	53.00	5.07	37.36

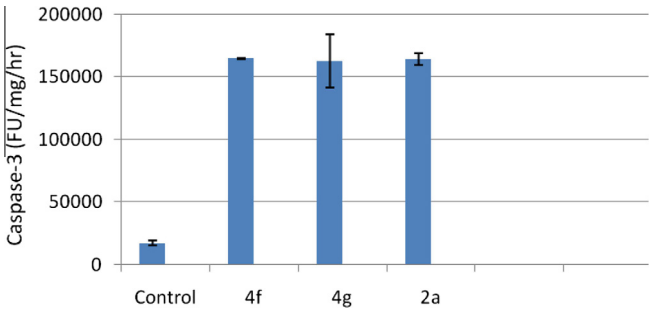


Figure 3. Effect of compounds **4f**, **4g** and **2a** on caspase-3 activity: HeLa cells were treated for 48 h with 2 μM concentrations of compounds **4f** and **4g** with **2a** as a positive control. Values indicate the mean SD of two different experiments performed in triplicates.

an IC_{50} value of 3.0 and 3.6 μM on the HeLa and ME-180 cell lines, while maintaining the same range of activity (1.9–5.1 μM) against DU-145, PC-3, COLO-205, HT-29 and B-16. Compound **5i**, with 3,4,5-tri-F substitution, showed IC_{50} range of 3.3–7.9 μM. Overall majority of the compounds in this series displayed better activity compared to its corresponding parent amine **16** against HeLa and ME-180 cell lines.

It is observed from these results that **4f** and **4g** with fluoro and trifluoromethyl groups located at 4-position on the cinnamide aromatic ring exhibited enhanced activity. The promising anticancer

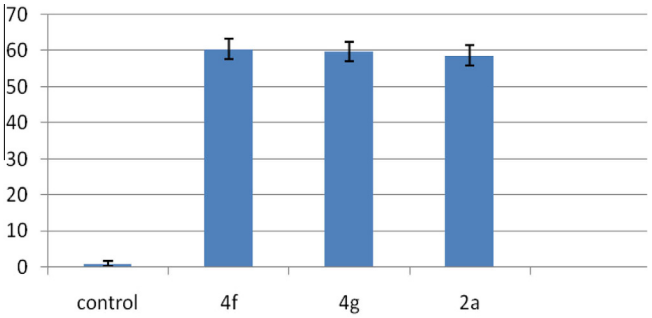


Figure 5. Effect on tubulin polymerization: tubulin polymerization assay was carried out in a reaction mixture that contained PEM buffer and GTP (1 mM) in the presence or absence of test compounds (**4f**, **4g**, and **2a**) at 5 μM concentration. The reaction was initiated by the addition of GTP to all the wells. Tubulin polymerization was monitored by the increase in fluorescence at 420 nm (excitation wavelength is 360 nm) was measured for 1 h at 1 min interval in a multimode plate reader (Tecan) at 37 °C.

activity showed by compounds **4f** and **4g** prompted us to evaluate their cell viability in the HeLa cells, with a view to study their detailed biological effects in this cell line.

In order to understand the role of these compounds (**4f**, **4g**, **2a**) in cell cycle, FACS analysis[22] was conducted in Cervical cancer cells (HeLa). The cells were treated for 24 h with compounds **4f**, **4g**, **2a** at 0.5 μM concentration. It was observed that HeLa cells showed 36.6%, 38.8%, 37.3% G2/M cell cycle arrest, respectively, whereas control (untreated cells) exhibited 21% as shown in Figure 2 and Table 3.

It is well known[23] that the cell cycle arrest at G2/M phase is shown to induce cellular apoptosis, hence it was considered of interest to examine whether the cytotoxicity of **4f**, **4g**, and **2a** is by virtue of apoptotic cell death. Cysteine aspartase group, namely, caspases play a crucial role in the induction of apoptosis and

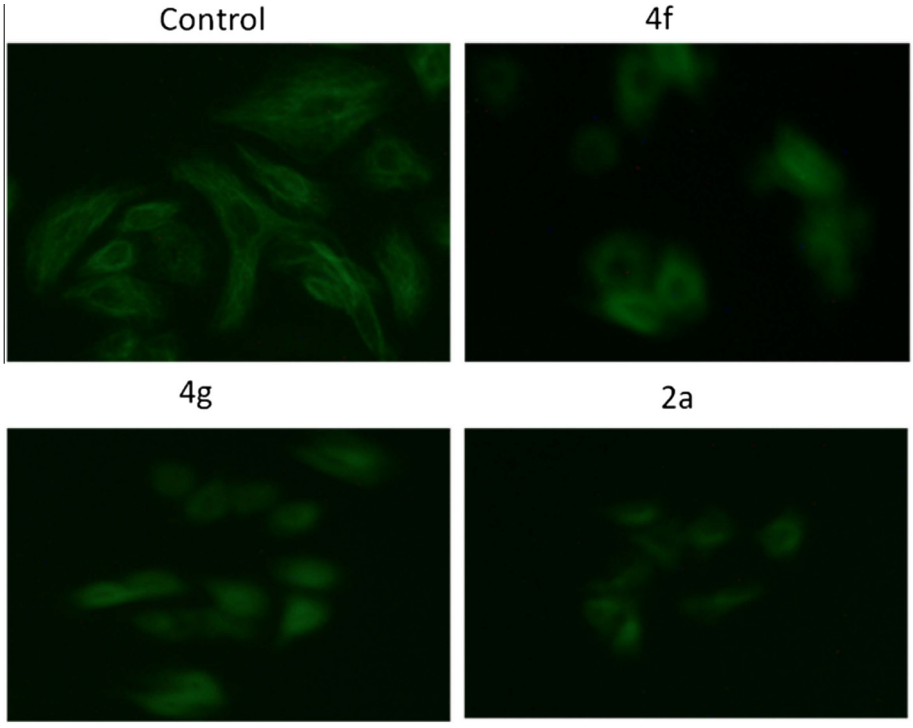


Figure 4. IHC analyses of compounds on the microtubule network: HeLa cells were treated with compounds **4f**, **4g**, and **2a** at 1 μM concentration for 48 h followed by staining with α-tubulin antibody. Microtubule organization was clearly observed by green color tubulin network like structures in control cells and was found to be disrupted in cells treated with compounds **4f** and **4g** with **2a** as positive control.

Table 4
Inhibition of tubulin polymerization (IC_{50})[a] of compounds **4f**, **4g** and **2a**

Compd	Tubulin polymerization inhibition $IC_{50} \pm SD$[b] (μM)
4f	0.6 ± 0.9
4g	0.7 ± 0.5
2a	**0.6 ± 0.3**

[a] Drug concentration required inhibiting 50% of tubulin assembly.
[b] Standard deviation.

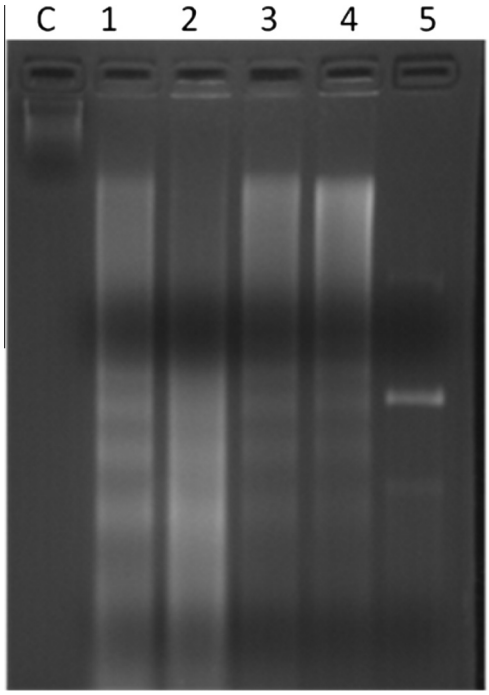


Figure 6. DNA fragmentations of compounds **4f** and **4g** in HeLa (Cervical cancer cells. Lane-1: (untreated control DNA (1 μl), lane-2: 1(**4f**-1 μM), lane-3: 2(**4f**-2 μM), lane-4: 3(**4g**-1 μM), lane-5; 4(**4g**-2 μM), lane-6: (5) marker, 50 bpair (loaded DNA for **4f** and **4g** at 1 μL).

amongst them caspase-3 happens to be one of the effector caspase. This prompted to treat HeLa cells with compounds, **4f**, **4g** and **2a** to examine the activation of caspase-3, The results indicate that there is nearly 5 to 8-fold induction in caspase-3 activity in cells treated with 2 μM concentration by these compounds as compared to control, thus suggesting the activation of caspase-3 by **4f**, **4g**, **2a** indicate that they have the ability to induce apoptosis in HeLa cells (Fig. 3).

In order to substantiate the observed effects of these compounds on the inhibition of tubulin polymerization to functional microtubules immunohistochemistry studies have been carried out to examine the in situ effects of compounds **4f** and **4g** on cellular microtubules in HeLa cancer cells. Therefore, HeLa cells were treated with **4f**, **4g**, and **2a** at 1 μM concentration for 48 h.[24] In this study, untreated human cervical cancer cells displayed the normal distribution of microtubules (Fig. 4). However, cells treated with compounds **4f** and **4g** showed disrupted microtubule organization as seen in Figure 4, thus demonstrating the inhibition of tubulin polymerization. This immunofluorescence study showed that the level of tubulin polymerization inhibition was comparable to that of **2a** for the compounds **4f** and **4g**.

One of the possibilities that these compounds exhibit anticancer activity as well as G2/M cell cycle arrest is by the inhibition of tubulin polymerization[25] as this has been observed in many antimitotic agents such as combretastatins. Hence it was considered of interest to investigate the tubulin polymerization aspect. As tubulin subunits heterodimerize and self-assemble to form microtubules in a time dependent manner, we have investigated the progression of tubulin polymerization by monitoring the increase in fluorescence emission at 420 nm (excitation wavelength is 360 nm) in 384 well plate for 1 h at 37 °C with and without the compounds at 5 μM concentration. The compounds **4f**, and **4g** inhibited tubulin polymerization by 60.5%, and 59.4%, respectively, compared to control, (Fig. 5). Tubulin polymerization inhibition was also observed in case of standards like, **2a** (58.3%).

Furthermore, these two potential compounds (**4f** and **4g**) were evaluated for their in vitro tubulin polymerization assay at different concentrations. These molecules showed potent inhibition of tubulin polymerization with IC_{50} values 0.6 and 0.7 μM, respectively, compared to the control **2a** having IC_{50} of 0.6 μM (Table 4).

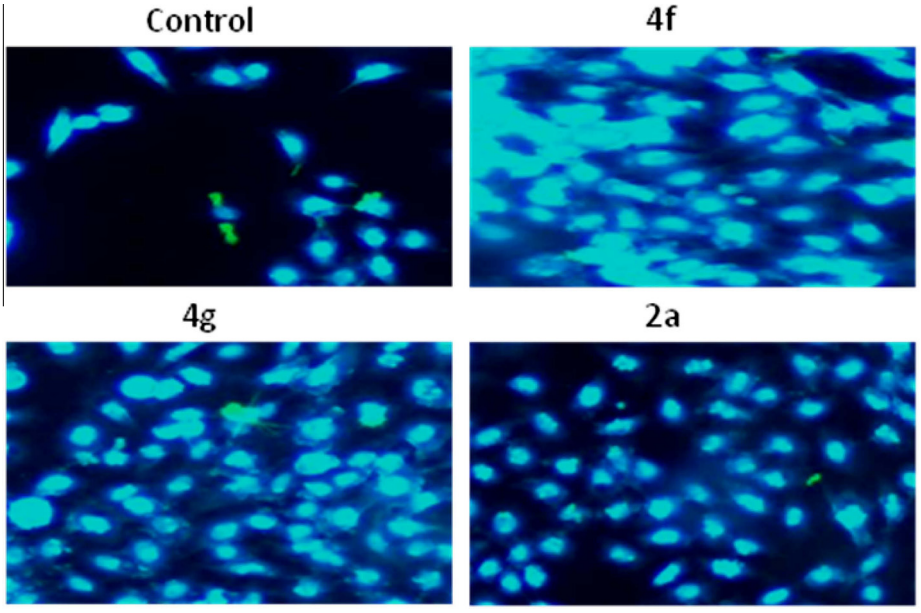


Figure 7. Hoechst staining in HeLa cervical cancer cell line, A: HeLa control cells, **4f**, **4g**, **2a** treated with 1 μM.

The ability of **4f** and **4g** for the induction of intranucleosomal DNA fragments at 48 h of exposure to HeLa cells have been demonstrated by agarose gel electrophoresis technique.[26] DNA laddering is significantly observed in the HeLa cells exposed to 1 and 2 μM of **4f** and **4g**. The results show that **4f** and **4g** harbor DNA fragmentation in HeLa cells, evident by Figure 6, which is associated with the last events of drug induced apoptosis.

Apoptosis is one of the major pathways that lead to the process of cell death. Chromatin condensation and fragmented nuclei are known as the classic characteristics of apoptosis. It was considered of interest to investigate the apoptotic inducing effect of these compounds (**4f** and **4g**) by Hoechst staining[23a] (H33258) method in HeLa cancer cell line. Therefore cells were treated with **4f**, **4g**, and **2a** at 1 μM concentrations for 48 h. Manual field quantification of apoptotic cells based on cytoplasmic condensation, presence of apoptotic bodies, nuclear fragmentation and relative fluorescence of the test compounds (**4f**, **4g**, and **2a**) revealed that there was significant increase in the percentage of apoptotic cells (Fig. 7).

A new series of benzophenone hybrids were synthesized and evaluated for their anticancer potential against panel of cancer cell lines. All these compounds exhibited cytotoxicity with IC_{50} values ranging from 0.06 to 16.3 μM and compounds **4f** and **4g**, bearing 4-F and 4-CF_3 on the cinnamido subunit, showed significant anticancer activity in HeLa cell line. Further the most active hybrids **4f** and **4g** induce apoptotic cell death by inhibition of tubulin polymerization leading to cell cycle arrest at G2/M phase of the cell cycle followed by caspase-3 activity. Hoechst 33258 staining and DNA fragmentation assay also suggests that **4f** and **4g** induces cell death by apoptosis. The in vitro tubulin polymerization assay showed that these compounds showed that the level of tubulin inhibition was comparable to that of **2a**. Based on these results it is evident that these new benzophenone–cinnamide hybrids, particularly **4f** and **4g**, have the potential to be developed as a new class of anticancer agents by further structural modifications. Overall, the current study demonstrates that the synthesis of cinnamido linked benzophenone conjugates as promising anticancer agents with G2/M arrest and apoptotic-inducing activities via targeting tubulin deserving further research and development, for exploiting new tubulin binding agents leading to apoptotic cell death.

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2014.03.076.

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