Novel HDAC/Tubulin Dual Inhibitor: Design, Synthesis and Docking Studies of α-Phthalimido-Chalcone Hybrids as Potential Anticancer Agents with Apoptosis-Inducing Activity

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Introduction: In order to develop novel anticancer HDAC/tubulin dual inhibitors, a novel series of α-phthalimido-substituted chalcones-based hybrids was synthesized and characterized by IR, ¹H NMR, ¹³C NMR, mass spectroscopy and X-ray analysis.

Methods: All the synthesized compounds were evaluated for their in vitro anticancer activity against MCF-7 and HepG2 human cancer cell lines using MTT assay. To explore the mechanism of action of the synthesized compounds, in vitro β-tubulin polymerization and HDAC 1 and 2 inhibitory activity were measured for the most potent anticancer hybrids. Further, cell cycle analysis was also evaluated.

Results: The trimethoxy derivative 7j showed the most potent anticancer activity, possessed the most potent β-tubulin polymerase and HDAC 1 and 2 inhibitory activity and efficiently induced cell cycle arrest at both G2/M and preG1 phases in the MCF-7 cell line.

Keywords: chalcones, α-phthalimido, anticancer, cell cycle, histone deacetylase inhibitor, tubulin polymerase inhibitor

Summary
- Novel series of α-phthalimido-chalcone hybrids were synthesized and identified by different spectroscopic techniques.
- The newly synthesized compounds experienced potent anticancer activity with IC₅₀ at micromolar concentrations against MCF-7 and Hep G2 cell lines, particularly, 7j hybrid exhibited 2.57 and 4.51 fold superior anticancer activity than CA-4 against the tested cell lines, respectively.
- The most potent anticancer compounds were investigated as HDAC/tubulin dual inhibitors.
- Compound 7j inhibit in vitro β-tubulin polymerization and HDAC 1 and 2 activity at IC₅₀ lower than that of CA-4 and entinostat.
- Cell cycle analysis was done for the most active compounds and it was clear that the tested compounds successfully arrested cell cycle at G2/M phase and induced preG1 apoptosis compared to the reference compounds.
- Molecular docking studies were carried out to explore the binding pattern of the most active synthesized compounds toward colchicine binding site in tubulin protein and HDAC2 active site.

Introduction
Cancer is basically a group of fatal diseases recognized by uncontrolled cell growth.¹ While chemotherapy remains the main treatment, clinically used anticancer drugs are
generally limited by toxicity, side effects, and resistance. Liver cancer (hepatocellular carcinoma, HCC), with globally rising prevalence remains one of the most common and lethal type of cancer. It ranks as the sixth most frequent cancer type and fourth most common cause of cancer-related death worldwide, as well as being responsible for more than 80% of diagnosed primary liver cancer worldwide. Moreover, the therapeutic options remain very limited and restricted on liver resection, transplantation and chemoembolization. Notably, a small proportion of patients are suitable candidates for these options and the relapse rate is high. On the other hand, breast cancer is the leading cause of death among cancer-related deaths in women, affecting 2.1 million women every year. Despite the recent introduction of several promising novel therapies that have shown considerable therapeutic success including hormone therapy, radiotherapy and chemotherapy, drug resistance remains one of the major challenges in breast cancer treatment.

Based on the aforementioned aspects, new curative therapeutic strategies are urgently needed that have the advantages of overcoming the anticancer therapy resistance and have superior efficacy, lower toxicity as well as better selectivity. Apart from these therapies, histone deacetylase inhibitors (HDACi) are epigenetic drugs, recently discovered, targeting specific parts of cancer cell signalling, particularly epigenome of cancer cells. Studies have shown that the impairment in the balance between the opposing actions of histone deacetylases (HDACs) and histone acetyltransferases (HATs) is responsible for epigenetic dysregulation of gene expression which results in development of many of cancer types. Among HDACs, HDAC2 is the most commonly overexpressed in many cancer types particularly HCC and breast cancers. Interestingly, HDACi can induce growth arrest in transformed cell, cell death and/or inhibition of angiogenesis, activation of the extrinsic and/or intrinsic apoptotic pathways and mitotic cell death. Fortunately, normal cells are relatively resistant to HDACi-induced cell death. Recently, several HDACi such as vorinostat, belinostat, panobinostat (hydroxamates), romidepsin (cyclic peptides), chidamide (benzamide) have been approved by FDA for treatment of cutaneous T-cell lymphoma, various hematological and solid cancers (Figure 1). The benzamide category can selectively inhibit class I HDACs (HDACs 1, 2, 3, and 8) by inducing cell cycle arrest and apoptosis of cancer cell lines, such as entinostat (MS-275) which is in Phase II clinical trial.

On the other hand, microtubules are polymers of α- and β-tubulin heterodimers which play the main role in regulating key cellular processes such as cell growth and division, preservation of the cell architecture and motility, spindle formation and intracellular trafficking. Consequently, due to these key roles tubulin targeting agents constitute an attractive class of chemotherapeutic drugs and it includes several agents, such as colchicine, combretastatin (CA-4), podophyllotoxin, vincristine (VCR), vinorelbine (VRB) and vinblastine (VBL) (Figure 2). CA-4 is a powerful antimicrototic compound, possessing potent anticancer activity against a number of human cancer cell lines involving multidrug resistant cancer types. It acts as a microtubule destabilizing agent by specifically binding to β-subunit of tubulin at the

![Figure 1 Examples of some approved HDACi.](https://www.dovepress.com/doi.pdf)
same site of colchicine and thus strongly inhibits tubulin polymerization.16

While searching for a new anticancer agent, chalcones have shown a successful design of effective antitumor agent. They are considered as one of the privileged scaffolds displaying multifarious applications in a wide variety of scientific domains, such as anticancer,17,18 anti-inflammatory,19 antioxidant,20 antimicrobial, anti-tubercular,21 anti-HIV,22 antimalarial,23 anti-allergic,24 and leishmanicidal activities.25 Notably, different mechanisms of action have been described for chalcones, including the induction of apoptosis, inhibition of cell proliferation, inhibition of angiogenesis, blockade of nuclear factor-kappa B (NF-κB) signaling pathway and reversal of multidrug resistance or a combination of these mechanisms.26,27 Furthermore, chalcones are powerful tubulin assembly inhibitors, with almost similar potency to CA-4.28

Of considerable interest, phthalimide and its derivatives have recently emerged as an important class of compounds which are used as the starting synthon for synthesis of diverse biologically active compounds such as anti-inflammatory,29 antioxidant,30 anti-angiogenic,31 hypoglycemic,32 antitumor,33 anti-tubercular agents34 as well as inhibitor of tumor necrosis factor-α (TNF-α) production.35 Moreover, α-phthalimido-ketones possess liver X receptor antagonistic,36 leukotriene D4 receptor antagonistic,37 anti-inflammatory,38 and angiogenesis inhibitor activities.39

Despite the continuous developments in anticancer drugs, new and more effective compounds are still required for this disease to be under control. These considerations and the above-mentioned findings prompted us to design a novel series of α-phthalimido-substituted chalcones-based hybrid scaffolds by incorporating the two entities in a single molecule via benzamido-link (Scheme 1) aiming at synergizing the anticancer activity and decreasing the human cytotoxicity by demonstrating their potential activity as promising HDAC-tubulin dual inhibitors. The novel hybrids were tested for their in vitro anticancer activity against certain human cancer cell lines. Moreover, the effects of these hybrids as inhibitors of histone deacetylase and tubulin polymerase were studied and supported with evaluation of the cell cycle analysis and apoptotic nature of the most potent compounds. Furthermore, molecular docking studies were also performed to investigate the interaction of inhibitors with the colchicine binding site of tubulin and HDAC2 active site (Figure 3).

Results and Discussion

Chemistry

The synthesis of the target compounds 7a-j is depicted in Scheme 1. The synthesis of compounds 4 and 5 followed the literature procedures40 using phthalimide as starting material. Reduction of phthalimide with zinc dust in the presence of copper sulphate followed by cyclization gave phthalide, which on reaction with potassium phthalimide in DMF resulted in formation of α-phthalimido-α-toluic acid (4). Treatment of 4 with thionyl chloride gave α-phthalimido-α-toluoyl chloride (5). N-(4-Acetylphenyl)-2-(1,3-dioxoisindolin-2-yl)benzamidine (6) has been synthesized by the reaction of 5 with 4-amino-acetophenone in the presence of triethyl amine (TEA) in 92% yield.

The structure of compound 6 was assigned on the basis of satisfactory elemental analysis as well as spectroscopic data. The FT-IR spectrum displayed two bands at 3454 and 3310 cm\(^{-1}\) due to NH, a band at 3097 cm\(^{-1}\) due to Ar-CH, a band at 2926 cm\(^{-1}\) due to Aliph-CH, and three bands at 1766, 1716, 1686 cm\(^{-1}\) due to CO, as well as a band at 1595 cm\(^{-1}\) due to C=N. The \(^1\)H NMR Spectrum of compound 6 indicated the

![Figure 2 Examples on some microtubule targeting agents with affinity for colchicine-binding site.](image-url)
presence of three singlets at 10.64, 5.03, 2.56 ppm, arising from NH, CH$_2$ and CH$_3$ resonances, respectively. Moreover, this interpretation is nicely confirmed by MS spectrum, in which the molecular ion peak and the base peak are the same, and appeared at m/z 398. The structure elucidation of compound 6 was also evidenced by X-ray crystallographic analysis of single crystal (Figure 4) (Supplementary Figure S1 and Table S1).

As illustrated in Scheme 1, compound 6 reacted with substituted aldehydes in the presence of sodium hydroxide under Claisen-Schmidt condensation conditions to form the chalcones 7a-j in 93–71% yield. The chemical structure of the target compounds were elucidated utilizing the spectral data and elemental analyses. The IR spectral data of compounds 7a-j showed intense peaks at 3468–3320 cm$^{-1}$ (NH) and 1733–1637 cm$^{-1}$ (CO). Another typical signal of these compounds was found in the $^1$H NMR spectra at 10.78–10.51 ppm (NH), however the aromatic as well as the olefinic protons appear as complexed multiplets in the region 9.15–6.11 ppm. The methylene protons resonate at 4.90–4.63 ppm. Additionally, the structure of 7a-j is confirmed by some $^{13}$C NMR by giving peaks at (CO-CH=CH) at 202.11–187.29 ppm, CO-N-CO at 186.11–169.44 ppm, CO-NH at 169.89–158.23 ppm, and CH$_2$ in compounds 7a-j resonate in the region of the solvent. The EI-MS spectra of 7a-e further support the given structure as they showed correct molecular ion peak.

**Biological Evaluation**

**In vitro Anticancer Activity Screening**

The novel synthesized hybrids were investigated for their in vitro anticancer activity against human MCF-7 (breast cancer) and human Hep G2 (hepatocellular liver carcinoma) cell
lines by using MTT assay. The CA-4 was used as the reference compound. The results are listed in Table 1 and graphically presented in Figure 5A and B.

The in vitro screening results revealed that the majority of the synthesized compounds displayed promising anticancer activity against the tested cancer cell lines at low
IC\textsubscript{50} values. In particular, trimethoxy hybrid 7j was the most active among the synthesized compounds with IC\textsubscript{50} values of 1.88 ± 0.06 µM and 1.62 ± 0.02 µM against both MCF-7 and Hep G2 cell lines, respectively; which represents 2.57 and 4.51 fold superior activity than the reference compound. It is noteworthy that, the dimethoxy derivative 7g and the \textit{p}-methoxy derivative 7c exhibited remarkable anticancer activity of 2.32 and 2.01 times the activity of CA-4 against MCF-7, where the IC\textsubscript{50} values were 2.09 ± 0.19 µM and 2.41± 0.11 µM, respectively. While, the anticancer potency of the two hybrids 7g and 7c against Hep G2 was nearly equipotent and nearly 4-fold the reference activity. Introduction of piperonyl group in 7i hybrid increased the anticancer activity by 1.4 and 3.73 fold compared to the reference compound against MCF-7 and Hep G2 cell lines, respectively.

The \textit{p}-chloro hybrid 7b showed slightly greater anticancer activity compared to CA-4 against MCF-7 cell line. However, this compound achieved IC\textsubscript{50} value of 3.21±0.17 µM which represents 2.28 time more potent anticancer activity than CA-4 against Hep G2 cell line. Meanwhile, the [2,2]paracyclophanyl 7e and 2-furyl 7h hybrids experienced nearly equal anticancer activity against both MCF-7 and Hep G2 cell lines, which was slightly weaker than the anticancer activity of CA-4 as indicated by their IC\textsubscript{50} values. According to their IC\textsubscript{50} values, the \textit{p}-nitro 7f and 1-naphthyl 7d hybrids had weak anticancer activity compared to CA-4. However, the anticancer activity markedly decreased in the case of the unsubstituted phenyl ring, which in turn confirmed that the substitution in the phenyl ring is critical for potentiating the anticancer activity. Also, the anticancer activity of compound 6 was dramatically decreased which emphasized the importance of the presence of the chalcone-based system. Finally, analyzing the above-mentioned results revealed that the methoxy derivatives experienced the most potent anticancer activity and this activity decreased in this order: tri-OCH\textsubscript{3} > di-OCH\textsubscript{3} > mono-OCH\textsubscript{3}. Of considerable interest, modification of the electronic nature of substituents attached to the phenyl ring of the chalcone moiety influenced significantly the anticancer activity. Where, the piperonyl 7i and \textit{p}-Cl-phenyl 7b derivatives possessed remarkable anticancer activity, the [2,2] paracyclophanyl 7e and 2-furyl 7h hybrids showed a slight decrease in the anticancer activity. Moreover, the anticancer activity was markedly decreased in the \textit{p}-NO\textsubscript{2}-phenyl 7f and 1-naphthyl 7d derivatives.

In vitro Enzyme Inhibition

In vitro Tubulin Polymerization Inhibitory Activity

To predict the possible mechanism of action of the synthesized compounds, \(\beta\)-tubulin (TUB\textsubscript{B}) polymerization inhibitory activity of the most potent synthesized hybrids 7c, 7g and 7j was investigated against the tested cancer cell lines. CA-4 was used as reference compound and the results are presented as IC\textsubscript{50} values (in µM concentrations) in Table 2 and Figure 6.

The results represented in Table 2 proved that 7j hybrid with IC\textsubscript{50} value of 2.32 ± 0.15 µM had the most potent \(\beta\)-tubulin polymerization inhibitory activity among tested and reference compounds. While 7g and 7c hybrids exhibited \(\beta\)-tubulin polymerization inhibition activity comparable to that of CA-4 which cleared by their IC\textsubscript{50} values. These results supported that 7j hybrid is a potent inhibitor of tubulin assembly which was in accordance with the in

\begin{table}[h]
\centering
\begin{tabular}{|l|c|c|}
\hline
\textbf{Compound No.} & \textbf{IC\textsubscript{50} (µM)} & \textbf{Hep G2} \\
\hline
6 & 17.59 ± 0.46 & 10.82 ± 0.63 \\
7a & 14.43 ± 0.51 & 12.93 ± 0.26 \\
7b & 4.11 ± 0.17 & 3.21 ± 0.17 \\
7c & 2.41 ± 0.11 & 1.87 ± 0.13 \\
7d & 11.31 ± 0.43 & 8.07 ± 0.39 \\
7e & 5.28 ± 0.25 & 8.47± 0.34 \\
7f & 9.78 ± 0.54 & 15.85± 0.71 \\
7g & 2.09 ± 0.19 & 1.75 ± 0.08 \\
7h & 5.44 ± 0.21 & 9.05 ± 0.33 \\
7i & 3.45 ± 0.27 & 1.96 ± 0.07 \\
7j & 1.88 ± 0.06 & 1.62 ± 0.02 \\
CA-4\textsuperscript{b} & 4.85 ± 0.18 & 7.32 ± 0.28 \\
\hline
\end{tabular}
\caption{IC\textsubscript{50} Values for Anticancer Activity of Compounds 6, 7a–7j and CA4 Using MTT Assay Against MCF-7, and HepG2 Cell Lines}
\end{table}
In vitro cytotoxic activity of these compounds against MCF-7 and Hep G2 cell line.

In vitro HDAC Inhibitory Activity
To further explore the mechanism of action of the synthesized compounds, compounds 7c and 7g and 7j were tested for in vitro HDAC inhibitory activity, IC_{50} values for the selected hybrids were determined against HDAC1 and 2 isoforms using entinostat as a reference compound (Table 3, Figure 7).

The results presented in Table 3 reveal that all the selected hybrids possessed potent HDAC1 and HDAC2 inhibitory activity. In particular, 7j hybrid afforded the highest HDAC1 and HDAC2 inhibitory activity with IC_{50} value lower than that of entinostat. Meanwhile, compound 7g exhibited nearly equipotent HDAC1 and HDAC2 inhibitory activity to entinostat which cleared by the proximity of their IC_{50} values. Moreover, both HDAC1 and HDAC2 inhibitory activities of compound 7e were slightly lower than entinostat IC_{50} values. In conclusion, the above-mentioned results evidenced that 7g and 7j hybrids are potent inhibitors of both HDAC1 and HDAC2. This was confirmed also by their potent in vitro cytotoxic activity against the tested cancer cell lines.

Table 2 IC_{50} Values for in vitro β-Tubulin Polymerization Inhibitory Activity of 7c, 7g, 7j, and CA-4 Using ELISA Assay

| Compound No. | Tubulin IC_{50} (μM)\(^{a}\) |
|--------------|--------------------------------|
| 7c           | 3.68 ± 0.21                    |
| 7g           | 3.27 ± 0.33                    |
| 7j           | 2.32 ± 0.15                    |
| CA-4\(^{b}\) | 2.62 ± 0.14                    |

Notes: \(^{a}\)Mean IC_{50} value of three independent experiments. \(^{b}\)CA-4: Positive reference.
In vitro Cell Cycle Analysis

In vitro DNA-Flow Cytometry

In order to further explore the effect of the most promising cytotoxic compounds on the cell cycle, cell cycle distribution using DNA flow cytometry analysis was assessed on 7j and 7c hybrids compared to CA-4 and entinostat (at their IC\textsubscript{50} concentration) against MCF-7 cell line. According to the results presented in Tables 4, 7j and 7g hybrids experienced the greatest activity by keeping cells in G2/M and preG1 phases compared to untreated control cell and the reference compounds (Figure 8A and B).

Moreover, the percentage of cells population at G2/M after 7j and 7g treatment was higher than entinostat by 1.53 and 1.42 folds, respectively. Also, 7j hybrid achieved 1.05 fold higher cell accumulation percentage than CA-4 at G2/M phase. While, 7c hybrid increased G2/M cells by nearly equal percentage to CA-4. Interestingly, it is clearly observed that 7j and 7g hybrids exhibited higher apoptosis ability than the untreated control cell as well as the reference compound entinostat at preG1 phase by 1.04 and 1.09 times, respectively.

These data established that the selected hybrids have potent anti-proliferative effects and apoptosis-inducing activity by increasing preG1 apoptosis and developing cell growth arrest at G2/M phase.

Table 3 IC\textsubscript{50} Values for in vitro HDAC1 & 2 Inhibitory Activity of Compounds 7c, 7g, 7j and Entinostat

| Compound No. | HDAC1 IC\textsubscript{50} (µM) | HDAC2 IC\textsubscript{50} (µM)\textsuperscript{*} |
|--------------|-------------------------------|----------------------------------|
| 7c           | 0.49 ± 0.03                   | 0.85 ± 0.06                      |
| 7g           | 0.32 ± 0.01                   | 0.56 ± 0.03                      |
| 7j           | 0.22 ± 0.01                   | 0.43 ± 0.02                      |
| Entinostat\textsuperscript{b} | 0.28 ± 0.02                   | 0.55 ± 0.04                      |

Notes: \textsuperscript{*}Mean IC\textsubscript{50} value of three independent experiments. \textsuperscript{b}Entinostat: Positive reference.

Annexin V-FITC/PI Staining Assay

It was obvious from cell cycle analysis results that the treatment of MCF-7 with the selected hybrids showed considerable increase in preG1 phase which is a suggestion of apoptosis (Table 5, Figure 9). Consequently, to determine the ability of 7j and 7g hybrids for inducing cell death in MCF-7 cell line via induction of apoptosis, Annexin V-FITC/PI staining assay was carried out for the selected compounds at their IC\textsubscript{50} (µM) for 24 hours and analyzed.

As shown in Table 5, compounds 7j and 7g have the ability for inducing apoptosis at early apoptotic, late apoptotic stages and cell necrosis development, in comparison to untreated controls. It was found that compounds 7j and 7g increased cell populations at the early apoptotic stage by 14.36- and 14.87-fold more than untreated control, respectively. While, 7j and 7g hybrids treatment significantly increased the late apoptotic cells population by 44.60 and 45.24 folds, respectively. Of considerable interest, the selected compounds possessed increasing in both early and late apoptotic cells compared to the reference compounds, CA-4 and entinostat. It is noteworthy that 7j and 7g hybrids displayed significant increase in cell necrosis by 2.09- and 2.76-fold, respectively, compared to the untreated control cell. Moreover, the necrosis percent of the selected hybrids were nearly equal to CA-4 and entinostat. These findings evidenced that the synthesized hybrids cause impairment of cell division with apoptosis-inducing activity and confirmed also that these hybrids can potentially inhibit HDAC and tubulin polymerase enzymes.

Molecular Docking Study

Docking at the Colchicine Binding Site in Tubulin Protein

Docking studies have been carried out to explore the binding ability of the most potent cytotoxic hybrids 7j,
Table 4 Cell Cycle Analysis of Compounds 7j, 7g, CA-4 and Entinostat Against MCF-7 Cell Line

| Compound No. | DNA Content | %G0-G1 | %S | %G2/M | %Pre-G1 | Comment |
|--------------|-------------|--------|----|-------|---------|---------|
| 7j           |             | 27.58  | 24.32 | 48.1  | 23.52  | PreG1 apoptosis and Cell growth arrest at G2/M |
| 7g           |             | 31.52  | 23.94 | 44.54 | 24.63  | PreG1 apoptosis and Cell growth arrest at G2/M |
| CA-4         |             | 27.43  | 26.72 | 45.85 | 29.03  | PreG1 apoptosis and Cell growth arrest at G2/M |
| Entinostat   |             | 43.29  | 25.41 | 31.3  | 22.43  | PreG1 apoptosis and Cell growth arrest at G2/M |
| Control      |             | 55.46  | 29.71 | 14.83 | 1.78   | -       |

7g and 7c with colchicine binding site of tubulin. MOE program was used for docking the selected hybrids and CA4 into the colchicine binding site using the X-ray crystallographic structure of tubulin-colchicine complex (PDB code: 1SA0) as tubulin protein template. The results of energy binding scores and binding interactions are depicted in Table 6 and Figure 10.

The energy binding scores recorded in Table 6 showed that the selected hybrids displayed higher energy binding scores than the reference ligand, consequently, better binding at the colchicine binding site. The highest energy binding score of $-9.10$ Kcal/mol was observed with the trimethoxy hybrid $7j$, followed by dimethoxy $7g$ and mono-methoxy $7c$ hybrids, respectively, which justified the cytotoxicity results and the estimated tubulin inhibitory activity. The binding interaction of $7j$ hybrid with colchicine binding site is shown in Figure 10B, where two hydrogen bonding interactions were formed with GLN 11 and GLY 144 with bond length of 3.30 Å and 2.80 Å, respectively. In addition to $\pi$-cation interactions with LYS 254 with bond length of 3.74 Å. Moreover, compound $7g$ achieved two $\pi$-hydrogen interactions with GLN 11 and ALA 12 with bond length of 4.23 Å and 3.63 Å, respectively. In addition, another $\pi$-$\pi$ interaction was formed with TYR 224 (bond length: 3.81 Å) Figure 10D. Meanwhile, one hydrogen bonding interaction was formed between NH group of $7c$ hybrid and ASP 329 (bond length: 2.87 Å) Figure 10F. The reference ligand CA4 showed one hydrogen bonding interaction with SER 140 (3.03 Å) and $\pi$-hydrogen interaction with GLY 144 (3.80 Å) Figure 10H.

Docking at the HDAC2 Active Site

In order to investigate the possible binding modes between the synthesized compounds and HDAC active site, the docking studies on the most potent cytotoxic compounds $7j$, $7g$ and $7c$ were performed using MOE program where the 3D structure of the HDAC active sites is available at RCSB Protein Data Bank, HDAC2 (PDB code: 4LXZ). Entinostat was used as reference compound. The results were expressed as docking energy and binding interactions with the active site of HDAC2 isoform (Table 7 and Figure 11).

Interestingly, the estimated docking energy of the selected compounds confirmed that the selected hybrids possessed higher binding energy than the reference compound which means better binding at the HDAC2 active site. Moreover, the order of binding energy was: tri-OCH$_3$ ($7j$) > di-OCH$_3$ ($7g$) > mono-OCH$_3$ ($7c$), which was consistent with the HDAC inhibition assay. Compound $7j$ showed an energy binding value of $-9.01$ Kcal/mol, while compounds $7g$ and $7c$ showed energy binding values of $-8.43$ and $-8.39$ Kcal/mol, respectively. Compound $7j$ showed two hydrogen bonding interactions between oxygen of both phthalimido- and chalcone-based moiety with ARG 197, ASN 331 of distances of 2.82 Å and 3.19 Å, respectively Figure 11B. In addition, one hydrogen bonding interaction was formed between oxygen of chalcone nucleus of $7g$ hybrid and ASN 331 of distance of 3.15 Å. Also, $\pi$-cation interaction was formed between phthalimido aromatic ring and NH$_2$ group of ARG 311 with distance of 5.00 Å Figure 11D. However, $7c$ hybrid exhibited one hydrogen bonding interaction with ASN 331 of 2.94 Å Figure 11F. On the other hand, entinostat showed one hydrogen bonding interaction with ASN 331 (3.09 Å) and another $\pi$-hydrogen interaction with PRO 344 (4.60 Å) similar to $7g$ hybrid and $7j$ hybrid, respectively Figure 11H.

Conclusion

A novel series of $\alpha$-phthalimido-substituted chalcones-based hybrids as dual HDAC/tubulin inhibitors was synthesized and evaluated for its in vitro anticancer activity by MTT method against MCF-7 and HepG2 human cell lines, using CA-4 as reference compound. Moreover, the in vitro tubulin polymerase and HDAC 1 and 2 inhibitory activity were evaluated for the most potent compounds $7c$, $7g$ and $7j$ using CA-4 and entinostat as reference compounds. The
Figure 8 (A) Cell cycle analysis in MCF-7 cell line treated with 7j and 7g hybrids at their IC$_{50}$ (µM). (B) Cell cycle analysis and apoptosis effect in MCF-7 cell line treated with 7j, 7g, CA-4 and entinostat compounds.
results revealed that the three substituted methoxy derivatives 7c, 7g and 7j experienced greater anticancer activity than that of CA-4; particularly the trimethoxy derivative 7j which experienced the most potent anticancer activity and potently inhibited β-tubulin polymerase and HDAC 1 and 2 activities. Additionally, the cell cycle analysis proved that these promising hybrids can induce apoptosis and prevent the proliferation of MCF-7 by sequestering higher cell percentage at G2/M and pre G1 phases than untreated control cells, CA-4 and entinostat. The molecular docking studies showed that these potent anticancer hybrids could efficiently bind to colchicine binding site of both tubulin polymerase enzyme and HDAC2 active sites with energy scores higher than the reference ligands. The results of our study proved that α-phthalimido-substituted chalcones-based hybrids may serve as novel promising dual HDAC/tubulin inhibitors in searching for more effective anticancer agents.

### Experimental Chemistry

Melting points were determined on Mel-Temp hot stage apparatus and were uncorrected. Reactions were routinely monitored by thin-layer chromatography on Merck silica gel PF₅₀ plates and spots were visualized with UV light at 254 nm. IR spectra were recorded as KBr disks on a Fourier Transform (FT-IR) Bruker spectrophotometer at Central Lab, Faculty of Science, Minia University, El-Minia, Egypt. ¹H NMR and ¹³C NMR spectra were carried out on a Bruker apparatus operating at 400 MHz and 100 MHz, respectively using TMS as internal reference at the Faculty of Science, Sohag University, Sohag, Egypt. Chemical shifts (δ values) are given in parts per million (ppm) using CDCl₃ (7.20) or DMSO-d₆ (2.5) as solvents and coupling constants (J) in Hertz. Splitting patterns are designated as follows: s, singlet; bs, broad singlet; d, doublet; t, triplet; m, multiplet. EI-MS: Mass spectrum was carried out on direct probe controller inlet part to single quadruple mass analyzer in (Thermo Scientific Gcms) Model (ISQ LT) using Thermo X-Caslibur Software at the Regional Center for Mycology and Biotechnology (RCMB) Al-Azhar University, Naser City, Cairo. Elemental analyses were performed on Perkin-Elmer 2400 CHN Elemental analyzer by the Microanalytical Center, Faculty of Science at the University of Cairo and are within ± 0.4% of theoretical values unless otherwise specified.

### Materials

Chemicals and solvents used in the preparation of the target compounds are of commercial grade, and purchased from Alfa Aesar, Cambrian chemicals, Aldrich, Acros Organics, Fluka, Merck, and El-Nasr Pharmaceutical Chemicals Companies. Chemicals were used without purification. Solvents were purified following the reported procedures.

#### Synthesis of α-Phthalimido-o-Toluoyl Chloride (5)

A mixture of potassium phthalimide, 50 g (0.26 mol) and 34.8 g phthalide (0.26 mol) in 150 mL DMF was refluxed for 5 hours, and then was cooled to r.t. and poured into a mixture of 100 mL glacial acetic acid and 150 mL water. The separated solid was washed with water and 95% ethanol. Recrystallization from acetic acid gave 41.35 g of α-phthalimido-o-toluic acid (4) (57%), m.p. 264–6°C, Lit. 40 266–7°C. A suspension of 50 g (0.178 mol) of 4 and 93 g (0.78 mol) thionyl chloride was refluxed for 3 hours. Dry benzene (30 mL) was added to the mixture and distilled with the excess thionyl chloride under reduced pressure. This later process was repeated twice to remove the excess thionyl chloride. The yield of the entited compound was 47.92 g (90%) as white prisms, m. p. 181–183°C, Lit. 40 183.5–184.8°C.

#### Synthesis of N-(4-Acetylphenyl)-2-(1,3-Dioxoisindolin-2-yl)Benzamide (6)

To a magnetically stirred solution of acid chloride 5 (5 g, 0.0167 mol) in abs. CH₂Cl₂ (20 mL) was added 4-aminoacetophenone (2.25 g, 0.0167 mol) in one portion and 1.68 g (0.0167 mol) TEA. The reaction mixture was stirred for a further 2 hours at r.t. and then treated with a saturated solution of NaCl (20 mL), and extracted with ethyl acetate (30 mL). The organic layer was washed with 1N HCl, NaHCO₃ solution and finally with H₂O, then dried over anhyd. Na₂SO₄. The solvent was removed under reduced pressure and the residue was recrystallized from ethyl acetate/n-hexane to give the entited compound.

White solid, 4.2 g (92%), m.p. 238–240 °C; FTIR (KBr, cm⁻¹) v: 3454, 3310 (NH), 3097 (Ar-CH), 2926 (Aliph.-CH), 1766, 1716, 1686 (CO), 1595 (C=N).

### Table 5 Results of Apoptotic Assay of 7j, 7g, CA-4 and Entinostat

| Compound No. | Apoptosis | Necrosis |
|--------------|-----------|----------|
|              | Total     | Early     | Late     |
| 7j           | 23.52     | 6.75      | 14.72    | 2.05     |
| 7g           | 24.63     | 6.99      | 14.93    | 2.71     |
| CA-4         | 29.03     | 4.92      | 21.65    | 2.46     |
| Entinostat   | 22.43     | 8.67      | 10.68    | 3.08     |
| Control      | 1.78      | 0.47      | 0.33     | 0.98     |
(Supplementary data page 1) $^1$H NMR (400 MHz, DMSO-$d_6$): $\delta$ 10.64 (s, 1H, NH), 7.95 (d, 2H, $J= 8.32$ Hz, Ar-H), 7.87 (d, 2H, $J= 8.32$ Hz, Ar-H), 7.84 (br. s, 4H, Ar-H), 7.61 (d, $J= 8.32$ Hz, 1H, Ar-H), 7.47 (t, $J= 8.32$ Hz, 2H, Ar-H), 7.42 (t, $J= 8.32$ Hz, 2H, Ar-H), 7.32 (d, $J= 8.32$ Hz, 1H, Ar-H), 5.03 (s, 2H, CH$_2$), 2.56 (s, 3H, CH$_3$) (Supplementary data page 12, 13). MS: m/z (rel. intensity), 399 (M$^+$, 1, 33), 398 (M$^+$, 100), 397 (19), 381 (11), 264 (22), 263 (9), 265 (4), 251 (2), 133 (4), 131 (6), 132 (3), 130 (54), 120 (15), 116 (21), 104 (11), 90 (7), 77 (20), 76 (20), 63 (3) (Supplementary data page 31). Anal. Calcd. for C$_{24}$H$_{18}$N$_2$O$_4$ (398.41): C, 72.35; H, 4.55; N, 7.03. Found: C, 72.05; H, 4.75; N, 6.82.

Synthesis of (E)-2-((1,3-Dioxoisindolin-2-yl)Methyl)-N-(4-(3-(Arylacroyloyl)Phenyl)Benzamides 7

General Procedure

A solution of 20% NaOH (0.5 mL) was added to a well
**Table 6** Energy Binding Scores (Kcal/mol) for Compounds 7j, 7g, 7c and CA4 with the Colchicine Binding Site

| Compound No. | Energy Binding Scores |
|--------------|-----------------------|
| 7j           | -9.10                 |
| 7g           | -8.87                 |
| 7c           | -7.20                 |
| CA4          | -6.76                 |

stirred mixture of compound 6 (0.16 g, 0.402 mmol) and different aldehydes (0.402 mmol) in 15 mL absolute ethanol at r.t. Stirring was continued for 8–20 hours. (The reaction was monitored by tlc). The reaction mixture was extracted with ethyl acetate, then treated with brine, 1N HCl, NaHCO₃, and washed with H₂O. The solvent was removed and the residue was recrystallized from dioxane-ethanol to afford the title compound.

(E)-N-((4-Cinnamoylphenyl)-2-((1,3-Dioxo-2,3-Dihydro-1H-Inden-2-Yl)Methyl)Benzamide (7a)
Pale yellow solid, 0.128 g (83%), m.p. 163–165 °C. FTIR (KBr, cm⁻¹) v: 3343 (NH), 3061 (Ar-(CH), 1700, 1653 (CO), 1600, 1523 (C=N) (Supplementary data page 2).

¹H NMR (400 MHz, DMSO-d₆): δ 10.74 (s, 1H, NH), 8.70–7.39 (m, 17 H, Ar-H, CH=CH), 6.65 (s, 2H, Ar-H), 4.65 (s, 2H, CH₂) (Supplementary data page 14, 15, 16);
³¹C NMR (100 MHz, DMSO-d₆): δ 188.24 (CO=CH), 169.44 (CONC), 168.38 (CONH), 168.29 (CO=CH), 143.62 (Ar-C), 138.99 (2Ar-C), 138.02 (Ar-C), 137.19 (Ar-C), 136.07 (Ar-C), 134.11 (Ar-C), 130.19 (Ar-C), 129.16 (2Ar-C), 129.02 (2Ar-C), 127.46 (Ar-C), 127.37 (Ar-C), 126.92 (Ar-C), 123.36 (2Ar-C), 122.69 (Ar-C), 119.97 (2Ar-C), 119.81 (2Ar-C), 115.02 (2Ar-C), 113.27 (2Ar-C) (Supplementary data page 42).

MS: m/z (rel. intensity) 487 (M⁺+1, 7), 486 (M⁺, 22), 486 (50), 453 (100), 250 (2), 133 (9), 120 (21), 117 (6), 104 (47), 103 (54), 97 (11), 92 (16), 78 (11), 77 (56), 65 (42), 51 (9) (Supplementary data page 32). Anal. Calcld. for C₃₁H₂₂N₂O₄ (486.52): C, 76.53; H, 4.56; N, 5.76. Found: C, 76.21; H, 4.72; N, 5.55.

(E)-N-4-(3-(4-Clorophenyl)Acryloyl)Phenyl)-2-((1,3-Dioxo-2,3-Dihydro-1H-Inden-2-Yl)Methyl)Benzamide (7b)
Yellow solid, 0.142 g (92%), m.p. 142–143 °C. FTIR (KBr, cm⁻¹) v: 3458, 3341 (NH), 3065 (Ar-(CH), 2924 (Aliph.-CH), 1702, 1653 (CO), 1601, 1523 (C=N) (Supplementary data page 3).

¹H NMR (400 MHz, DMSO-d₆): δ 10.75 (s, 1H, NH), 8.22–7.39 (m, 17 H, Ar-H, CH=CH), 6.66 (s, 1H, Ar-H), 4.64 (s, 2H, CH₂) (Supplementary data page 17, 18).

(E)-2-((1,3-Dioxo-2,3-Dihydro-1H-Inden-2-Yl)Methyl)-N-(4-(3-(4-Methoxyphenyl)Acryloyl)Phenyl)Benzamide (7c)
White solid, 0.115g (74%), m.p. 191–193 °C. FTIR (KBr, cm⁻¹) v: 3422, 3326 (NH), 3066 (Ar-(CH), 1704, 1678, 1654 (CO), 1597, 1516 (C=N) (Supplementary data page 4).

¹H NMR (400 MHz, DMSO-d₆): δ 10.73 (s, 1H, NH), 8.80–7.02 (m, 18H, Ar-H, CH=CH), 4.63 (s, 2H, CH₂), 3.85 (s, 3H, OCH₃) (Supplementary data page 19);
³¹C NMR (100 MHz, DMSO-d₆): δ 197.02 (CO=CH), 169.43 (CONC), 168.55 (CONH), 168.45 (CO=CH), 158.19 (Ar-C), 144.86 (Ar-C), 144.66 (Ar-C), 138.49 (Ar-C), 137.99 (Ar-C), 136.33 (2Ar-C), 136.19 (2Ar-C), 132.19 (Ar-C), 131.07 (Ar-C), 130.81 (Ar-C), 130.05 (2Ar-C), 129.88 (2Ar-C), 129.42 (2Ar-C), 129.07 (2Ar-C), 120.35 (Ar-C), 119.22 (Ar-C), 119.71 (2Ar-C), 114.55 (2Ar-C), 55.87 (OCH₃) (Supplementary data page 43).

Anal. Calcld. for C₃₃H₂₃ClN₂O₅ (516.54): C, 74.41; H, 4.68; N, 5.42. Found: C, 74.20; H, 4.33; N, 5.77.

(E)-2-((1,3-Dioxo-2,3-Dihydro-1H-Inden-2-Yl)Methyl)-N-(4-((Naphthyl-1-Yl)Acryloyl)Phenyl)Benzamide (7d)
Pale orange solid, 0.141g (91%), m.p. 141–142 °C. FTIR (KBr, cm⁻¹) v: 3328, 3320 (NH), 3046 (Ar-(CH), 1686, 1639 (CO), 1602, 1584 (C=N) (Supplementary data page 5).

¹H NMR (400 MHz, DMSO-d₆): δ 10.77 (s, 1H, NH), 9.15–7.42 (m, 20H, Ar-H, CH=CH), 6.69 (s, 1H, Ar-H), 4.65 (s, 2H, CH₂) (Supplementary data page 20).

MS: m/z (rel. intensity) 537 (M⁺+1, 5) 536 (M⁺, 18), 526 (36), 512 (10), 491 (12), 461 (2), 434 (7), 413 (10), 411 (100), 409 (23), 402 (16), 383 (15), 367 (3), 455 (2), 279 (2), 258 (30), 175 (3), 152 (18), 126 (3), 105 (2), 79 (4) (Supplementary
Figure 10 Docking and binding pattern of compounds 7j, 7g, 7c and CA-4 showing interactions with different amino acid residues found in the active site of colchicine binding site in tubulin protein (PDB code: 1SAO). (A) 3D structure of compound 7j (yellow) (B) 2D structure of compound 7j (C) 3D structure of compound 7g (yellow) (D) 2D structure of compound 7g (E) 3D structure of compound 7c (yellow) (F) 2D structure of compound 7c. (G) 3D structure of reference compound CA-4 (yellow) (H) 2D structure of reference compound CA-4.
Table 7 Energy Binding Scores (Kcal/mol) for Compounds 7j, 7g, 7c and Entinostat at the Active Site of HDAC2

| Compound No. | Energy Binding Scores |
|--------------|-----------------------|
| 7j           | -9.01                 |
| 7g           | -8.43                 |
| 7c           | -8.39                 |
| Entinostat   | -7.67                 |

Data page 35. Anal. Caled. for C$_{38}$H$_{32}$N$_2$O$_4$ (536.58): C, 78.34; H, 4.51; N, 5.22. Found C, 78.54; H, 4.51; N, 5.39.

(E)-2-((1,3-Dioxo-2,3-Dihydro-1H-Inden-2-Yl) Methyl)-N-(4-(3-[(2,2)Paracyclophan-4-Yl)Acryloyl]Phenyl)Benzamide (7E)

Pale yellow solid, 0.144g (93%), m.p. 153–154 °C. FTIR (KBr, cm$^{-1}$): ν 3411 (NH), 1711, 1647 (CO), 1601, 1530 (C=N) (Supplementary data page 6). $^1$H NMR (400 MHz, DMSO-d$_6$): δ 10.71 (s, 1H, NH), 8.07–7.41 (m, 17H, Ar-H, CH=CH), 4.64 (s, 2H, CH$_2$), 3.17–2.95 (m, 8H, 2 CH$_2$-CH$_3$) (Supplementary data page 21). MS: m/z (rel. intensity), 645 (M$^+$_1, 6), 644 (M$^+$, 5), 425 (8), 353 (15), 351 (15), 292 (5), 286 (7), 247 (8), 245 (9), 238 (5), 236 (12), 234 (7), 210 (7), 207 (15), 203 (8), 160 (6), 152 (4), 144 (11), 131 (18), 112 (10), 104 (100), 103 (99), 100 (15), 89 (16), 78 (60), 72 (27), 63 (9), 52 (19) (Supplementary data page 36). Anal. Caled. for C$_{43}$H$_{36}$N$_2$O$_4$ (644.76): C, 70.80; H, 5.63; N, 5.69. Found C, 70.83; H, 5.62; N, 5.98.

(E)-2-((1,3-Dioxo-2,3-Dihydro-1H-Inden-2-Yl) Methyl)-N-(4-(3-(4-Nitrophenyl)Acryloyl)Phenyl)Benzamide (7g)

Yellow solid, 0.115 g (74%), m.p. 162–164 °C. FTIR (KBr, cm$^{-1}$): ν 3421 (NH), 2933, 2839 (Aliph.-CH), 1713, 1653 (CO), 1596, 1506 (Aliph.-CH) (Supplementary data page 8). $^1$H NMR (400 MHz, DMSO-d$_6$): δ 10.71 (s, 1H, NH), 8.07–7.41 (m, 17H, Ar-H, CH=CH), 4.64 (s, 2H, CH$_2$), 3.17–2.95 (m, 8H, 2 CH$_2$-CH$_3$) (Supplementary data page 21). MS: m/z (rel. intensity), 547 (M$^+$_1, 4), 546 (M$^+$, 2), 493 (2), 485 (2), 446 (11), 430 (8), 428 (12), 414 (46), 400 (36), 382 (88), 357 (3), 340 (7), 314 (3), 308 (5), 283 (5), 262 (7), 254 (9), 236 (2), 250 (5), 190 (3), 183 (4), 178 (5), 166 (5), 163 (20), 149 (25), 133 (9), 121 (21), 104 (9), 92 (13), 76 (100), 69 (16), 65 (21), 63 (17) (Supplementary data page 38). Anal. Caled. for C$_{32}$H$_{28}$N$_2$O$_6$ (546.57): C, 72.52; H, 4.79; N, 5.13. Found C, 72.21; H, 4.58; N, 5.37.

(E)-2-((1,3-Dioxo-2,3-Dihydro-1H-Inden-2-Yl) Methyl)-N-(4-(3-(4-Dimethoxyphenyl)Acryloyl)Phenyl)Benzamide (7h)

Pale yellow solid, 0.109g (71%), m.p. 170–172 °C. FTIR (KBr, cm$^{-1}$): ν 3411 (NH), 1711, 1647 (CO), 1601, 1530 (Aliph.-CH) (Supplementary data page 9). $^1$H NMR (400 MHz, DMSO-d$_6$): δ 10.72 (s, 1H, NH), 8.30–6.66 (m, 17H, Ar-H, furyl), 4.90 (s, 2H, CH$_2$) (Supplementary data page 26, 27); $^{13}$C NMR (100 MHz, DMSO-d$_6$): δ 202.11 (COCH=CH), 186.11 (CONCO), 169.89 (CONH), 147.33, 146.73, 139.52, 138.18, 138.02, 135.09, 134.11, 130.00, 128.42, 128.22, 126.82, 124.33, 120.87, 119.89, 119.77, 116.82, 115.90, 114.61, 114.11 (Ar-C, furyl-C, CH=CH) (Supplementary data page 45). MS: m/z (rel. intensity), 477 (M$^+$_1+1, 4) 476 (M$^+$, 7), 475 (45), 474 (30), 473 (100), 458 (26), 456 (57), 440 (12), 264 (13), 213 (3), 195 (2), 185 (2), 159 (5), 156 (7), 133 (4), 130 (12), 120 (29), 105 (2), 102 (7), 92 (21), 76 (7), 65 (44) (Supplementary data page 39). Anal. Caled. for C$_{29}$H$_{20}$N$_2$O$_4$ (476.48): C, 73.10; H, 4.23; N, 5.88. Found C, 72.72; H, 4.55; N, 6.09.
Figure 11 Docking and binding pattern of compounds 7j, 7g, 7c and CA-4 showing interactions with different amino acid residues found in HDAC2 active site (PDB code: 4LXZ). (A) 3D structure of compound 7j (yellow) (B) 2D structure of compound 7j. (C) 3D structure of compound 7g (yellow) (D) 2D structure of compound 7g. (E) 3D structure of compound 7c (yellow) (F) 2D structure of compound 7c. (G) 3D structure of entinostat reference compound (yellow) (H) 2D structure of entinostat reference compound.
(E)-N-4-((3-(Benzo[D][1,3]Dioxol-5-Yl)Acryloyl)Phenyl)-2-((1,3-Dioxoisindolin-2-Yl)Methyl)Benzamid (7i)
Pale yellow solid, 0.114 g (74%), m.p. 244–246 °C. FTIR (KBr, cm⁻¹) v: 3450, 3349, 3241 (NH), 2908 (Aliph.-CH), 1642 (CO), 1588, 1552 (C=N) (Supplementary data page 10).
1H NMR (400 MHz, DMSO-d₆): δ 10.51 (s, 1H, NH), 7.91–6.43 (m, 15H, Ar-H, CH=CH), 6.11 (s, 2H, Ar-H), 4.59 (s, 2H, CH₂) (Supplementary data page 28, 29); MS. m/z (rel. intensity) 531 (M⁺+1, 2), 530 (M⁺, 20), 518 (13), 516 (2), 500 (15), 486 (23), 453 (3), 268 (40), 267 (100), 239 (19), 180 (7), 152 (6), 144 (28), 133 (6), 120 (36), 117 (9), 104 (15), 89 (48), 77 (19), 65 (61) (Supplementary data page 40). Anal. Calcd. for C₃₂H₂₂N₂O₆ (530.53): C, 72.45; H, 4.18; N, 5.28. Found C, 70.51; H, 5.11; N, 4.65.

Supplementary data page 11

Yellow solid, 0.181 g (78%), m.p. 187–189 °C. FTIR (KBr, cm⁻¹) v: 3454 (NH), 2932, 2853 (Aliph.-CH), 1733, 1637 (CO), 1563, 1538 (Aliph.-CH) (Supplementary data page 11).
1H NMR (400 MHz, DMSO-d₆): δ 10.69 (s, 1H, NH), 8.57–6.61 (m, 16H, Ar-H, CH=CH), 4.61 (s, 2H, CH₂), 3.89 (s, 3H, 1OCH₃), 3.85 (s, 6H, 2 OCH₃) (Supplementary data page 30); MS. m/z (rel. intensity) 576 (M⁺, 12), 462 (8), 444 (8), 430 (9), 416 (55), 409 (12), 383 (87), 355 (2), 340 (9), 312 (8), 297 (3), 279 (8), 264 (4), 236 (5), 193 (4), 167 (6), 160 (6), 132 (9), 104 (11), 76 (100), 77 (34) (Supplementary data page 41). Anal. Calcd. for C₃₄H₂₆N₂O₆ (576.60): C, 70.82; H, 4.89; N, 4.86. Found C, 70.51; H, 4.33; N, 5.47.

(E)-2-((1,3-Dioxoisindolin-2-Yl)Methyl)-N-((4-(3,4,5-Trimethoxyphenyl)Acryloyl)Phenyl)Benzamide (7j)

In vitro Enzyme Inhibitory Assays
To investigate the effect of synthesized hybrids on the enzymatic activity of HDAC and tubulin, MCF-7 line was used and incubated in DMEM supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin and 10 μg/mL insulin (Sigma). All of the other chemicals and reagents were from Sigma, or Invitrogen Cancer cells were seeded at density 1.2 x 10⁵/well in 100 μL DMEM using 96-well plates. To each well, 100 μL of the synthesized compounds 7c, 7g and 7j were added 18–24 hours prior to HDAC and tubulin inhibition assay.

Tubulin Polymerization Inhibitory Assay
The assay was done on MCF-7 cell line using TUbB ELISA kit (Cloud-Clone Corp.). Cancer cells were seeded into 96-well microtiter plates and kept in a humidified atmosphere at 37°C for 1 day. After dilution to the specified concentration, 100 μL of the synthesized hybrids 7c, 7g and 7j and CA4 were added to each well, then the plate was incubated for 2 hours at 37°C.

After discarding the supernatant, 100 μL of kit detection reagent A was added to each well, then the plate was re-incubated for 2 hours at 37°C. Kit detection reagent B was added to each well and the plate incubated for 30 minutes at 37°C. After five washing cycles, 90 μL of 3,3',5,5'-tetramethylbenzidine (TMB) solution was added to each well, then the plate was incubated for 15–25 minutes. 200 μL of 5% MTT reagent solution was added to each well plate and incubated for 3 hours. After an incubation period, the formed formazan crystals were dissolved in 200 μL isopropanol/well with continuous shaking using MaxQ 2000 plate shaker (Thermo Fisher Scientific Inc, MI, USA) at room temperature. Absorbance was measured spectrophotometrically at wavelength 570 nm using a Stat FaxR 4200 plate reader (Awareness Technology, Inc., FL, USA).
minutes at 37°C, followed by the addition of 50 μL of stop solution. Different concentrations (0.01, 0.1, 1, 10 μM) of the synthesized hybrids 7c, 7g and 7j and CA4 were incubated with the cells for 72 hours.

Absorbance was measured spectrophotometrically at wavelength 450 nm using a Stat FaxR 4200 plate reader (Awareness Technology, Inc., FL, USA). The concentration that inhibits 50% of maximum TUBB activity were determined and expressed as IC50 using Graph Pad Prism 5 software (Graph Pad software Inc, CA, USA).

**HDAC Inhibitory Assay**

The effect of the synthesized hybrids 7c, 7g and 7j on HDAC (1 and 2) were investigated using ELISA assay kit (Mybiosource, Inc.). According to manufacturer’s instructions, MCF-7 cell was seeded into 96-well microtiter plates and kept in a humidified atmosphere at 37°C for 1 day. After dilution to the specified concentration, 100 μL of the synthesized compounds 7c, 7g and 7j and positive control entinostat were added to each well, then the plate incubated for 2 hours at 37°C.

After discarding the supernatant, 100 μL of kit detection reagent A was added to each well, then the plate re-incubated for 2 hours at 37°C. Kit detection reagent B was added to each well and the plate incubated for 30 minutes at 37°C. After five washing cycles, 90 μL of 3,3′,5,5′-tetramethylbenzidine (TMB) solution was added to each well, then the plate was incubated for 15–25 min at 37°C, followed by the addition of 50 μL of stop solution. Different concentrations (0.01, 0.1, 1, 10 μM) of the synthesized hybrids 7c, 7g and 7j and entinostat were incubated with the cells for 72 hours.

Absorbance was measured spectrophotometrically at wavelength 405 nm using a Stat FaxR 4200 plate reader (Awareness Technology, Inc., FL, USA). The concentration that inhibits 50% of maximum HDAC activity were determined and expressed as IC50 using Graph Pad Prism 5 software (Graph Pad software Inc, CA, USA).

**In vitro DNA Flow Cytometry Assay**

MCF-7 cells at density of 2 x 10⁶/well were incubated with synthesized compounds 7g, 7j in addition to CA-4 and entinostat at their IC50 in DMSO (1% v/v). The cell plates were incubated for 24 hours. The cells were then fixed with 70% ice-cold ethanol after washing twice with cold phosphate buffered saline (PBS). Cells washed with PBS for 30 minutes at 37°C and recovered by centrifugation at 2000 rpm for 5 minutes.

Cells were stained with DNA fluorochrome propidium iodide (PI). The plates were incubated at room temperature in a dark place for 20 minutes. Then the cells were investigated with a FACS Caliber flow cytometer (Becton Dickinson, Heidelberg, Germany).

**Annexin V-FITC/PI Staining Assay**

MCF-7 cells at a density of 4 x 10⁶/well were incubated with synthesized compounds 7g, 7j in addition to CA-4 and entinostat at their IC50 for 24 hours. Cells were subjected to three washing cycles with ice cold PBS, then suspended in PBS. Cell apoptosis was detected by Annexin V-FITC Apoptosis Detection Kit (BioVisionResearch Products, USA). The cells were stained with PI staining solution, Annexin V-FITC and incubated at room temperature for 15 minutes in a dark place. Cells were investigated by flow cytometry using FACS caliber (Becton Dickinson, Heidelberg, Germany).

**Molecular Docking Study**

Molecular modelling studies of the selected derivatives 7c, 7g, 7j, CA-4 and entinostat were performed using MOE software. Ligands were built into the builder interface of the MOE program and their energies were minimized until a root mean square deviation (RMSD) gradient of 0.01 kcal/mol and a root mean square (RMS) distance of 0.1 Å with MMFF94X (Merck molecular force field 94X) forcefield and the partial charges were automatically calculated. The X-ray crystallographic structure of tubulin-colchicine complex (PDB code: 1SA0) and HDAC2 active site (PDB code: 4LXZ) were downloaded from protein data bank (www.rcsb.org). The enzymes were prepared, the hydrogens were added then the atoms connection and type were checked with automatic correction. The obtained poses were stored and the poses showing the best ligand-enzyme interactions were selected and stored for energy calculations.

**Crystal Structure Determinations**

The crystal was mounted in inert oil and transferred to the cold gas stream of a Rigaku-Oxford XtaLAB Synergy diffractometer using mirror-focussed Cu Kα radiation. Absorption corrections were based on multi-scans. The
structure was refined anisotropically on F² using the program
SHELXL-2017.45 The hydrogen atom of the NH group was
refined freely. Other hydrogens were included using a
riding model or rigid methyl groups. Complete
crystallographic data have been deposited at the Cambridge
Crystallographic Data Centre as CCDC 1,948,416.

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The authors report no conflicts of interest in this work.

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