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One-pot synthesis of cinnamylideneacetophenones and their *in vitro* cytotoxicity in breast cancer cells

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Abstract

A series of cinnamylideneacetophenones were synthesized via a modified Claisen-Schmidt condensation reaction and evaluated for cytotoxicity against breast cancer cells using the Alamar Blue™assay. Derivatives **17** and **18** bearing a 2-nitro group on the B ring, exhibited sub-micromolar cytotoxicity in MCF-7 cells (IC_{50} = 71 and 1.9 nM) respectively. Derivative **17** also displayed sub-micromolar (IC_{50} = 780 nM) cytotoxicity in MDA-MB-468 cells. Additionally, **17** and **18** displayed significantly less cytotoxicity than the chemotherapeutic doxorubicin in non-tumorigenic MCF-10A cells. This study provides evidence supporting the continued development of nitro-substituted cinnamylideneacetophenones as small molecules to treat breast cancer.

Keywords

breast cancer; estrogen receptor; chalcone derivatives; leinamycin; cytotoxicity

Despite current advances in therapy, breast cancer causes nearly 40,000 deaths each year in the US.[1] As a result, it is evident that more effective therapy is needed to treat these malignancies. Historically, breast cancer has been classified broadly as endocrine receptor

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(i.e. estrogen receptor or progesterone receptor) positive or negative. This stratification frequently serves as a guide for targeted therapy. Breast cancer that is estrogen receptor positive (ER+) and progesterone receptor positive (PR+) is frequently treated with hormone-based therapeutic agents such as the anti-estrogen tamoxifen (Nolvadex®) or aromatase inhibitors such as anastrazole (Arimidex®).[2] Despite their effectiveness, tamoxifen can cause thromboembolisms and endometrial cancer; while, anastrazole therapy increases the risk of arthralgia and bone fracture. In addition, there is a risk of hormone therapy-related resistance.[3–5]

More recently, breast cancer has included a classification for tumors that overexpress human epidermal growth factor receptor 2 (HER2) irrespective of ER/PR status. Such patients are frequently candidates for a targeted monoclonal antibody trastuzumab (Herceptin®). However, only about 25% of HER2 positive tumors respond to trastuzumab and a substantial percentage of these tumors acquire resistance to this agent. Interestingly, those that use this agent are at risk for developing cardiotoxicity.[6]

Tumors that lack appreciable expression of ER, PR and HER2 are denoted as ‘triple negative’ and are unresponsive to hormone-based or targeted therapeutic agents. As a result, patients bearing such tumors are limited to chemotherapy protocols with cytotoxic (non-specific) agents such as doxorubicin (Adriamycin®, DOXIL®), cyclophosphamide (Cytoxan®), and paclitaxel (Taxol®, Abraxane®), which are associated with severe adverse effects. Specifically doxorubicin can cause cardiotoxicity and secondary malignancies.[7] Considering the risk of hormone therapy-related resistance, the ineffectiveness of endocrine therapy in triple negative breast cancer and the severe side effects of nonspecific chemotherapeutic agents, a need persists for novel small molecules with the potential to effectively manage different breast cancer subtypes.

There have been a number of small molecules isolated and synthesized in hopes of discovering the next pharmacophore to serve as a template for effective agents to treat cancer, particularly breast cancer. Leinamycin, a naturally occurring antibiotic containing extended conjugation shows antitumor activity and is reported to cleave DNA.[8] Leinamycin exhibits potent anticancer activity both in cancer cell lines and in human tumor xenografts.[9] At least a portion of leinamycin’s anticancer activity resides within the disulfide bond of the 1,2-dithiolan-3,1-oxide heterocycle molecule which readily promotes reactive oxygen species formation and DNA damage.[8, 10] Although a significant amount of antitumor activity may exist in this portion of the molecule, it may not account for all of its antitumor actions.

Another class of anti-cancer compounds that have been extensively synthesized are chalcones (1,3-diaryl-2-propen-1- ones), flavonoid small molecules that possess a broad pharmacological profile including antioxidant, anti-inflammatory and anticancer actions.[11] In addition, these naturally occurring compounds disrupt mitosis and inhibit tubulin polymerization.[12] While the unsubstituted chalcone **2** does not occur naturally, polyhydroxylated chalcones and flavonoids exist in a myriad of edible plants.[13, 14] Structurally, chalcones are characterized by two aromatic rings joined by a three-carbon α,β-unsaturated carbonyl system.[15] The stilbene configuration within the α,β-unsaturated

carbonyl system has been used as a combinatorial starting point for novel medicinal entities and may also contribute to their antitumor properties. Stilbene derivatives include tamoxifen and the naturally occurring flavonoid genistein.[16]

We speculate that leinamycin's α,β,γ,δ–unsaturated carbonyl group, as highlighted in Figure 1, may also contribute to its anticancer activity. As a result, we designed, synthesized and evaluated a series of cinnamylideneacetophenones, or (E,E)-1,5- diarylpenta-2,4-dien-1-ones, with aryl substitution similar to published chalcones to determine if this portion of the molecule also contributes to their anticancer activity (Figure 1). Recent studies have extensively characterized certain cinnamylideneacetophenones though little is known about their potential to display anticancer activity.[17]

Using a Claisen-Schmidt condensation reaction, we initially synthesized a library of chalcones. We are reporting the two derivatives (**4** and **5**) with the most biological activity. Chalcone **4** has a tri-methoxy phenyl substitution on the A-ring and chalcone **5** bears a 2-nitro group on the B-ring (Scheme 1). We screened these chalcones using the Alamar Blue™ assay to compare their cytotoxicity levels with the unsubstituted trans-chalcone (**6**), which was purchased from Sigma-Aldrich® (CAS Number 614-47-1, Catalog Number 136123). We initially evaluated the anticancer activity of **4** and **5** in comparison to **6** and later to their corresponding cinnamylideneacetophenones to determine the impact of extending the conjugation on cytotoxicity in MCF-7 ER+ and MDA-MB-468 ER- breast cancer cells. We also evaluated their potential cytotoxicity in a non-tumorigenic breast epithelial cell line (MCF-10A).

Chalcone **4** demonstrated activity in the MDA-MB-468 cell line in the micromolar range while chalcones **5** and **6** were inactive. Both chalcones **4** and **5** displayed activity in MCF-7 cells while chalcone **6** was inactive in this cell line. All three chalcones displayed substantial toxicity in the MCF-10A non-cancerous cell line to suggest their high potential for toxicity in non-target cells (Table 1).

Following the construction of the chalcone analogues, we designed and synthesized a library of cinnamylideneacetophenones using a one-pot modified Claisen-Schmidt condensation reaction. The synthesis of cinnamylideneacetophenones **9–18** was accomplished by reacting trans-cinnamaldehydes containing varying functional groups with either acetophenone or 2-nitro acetophenone in the presence of aqueous NaOH and ethanol (Scheme 2). This library combines the common aryl groups of reported chalcones including those of **4** and **5**, with the extended conjugation and increased carbon length seen in the aforementioned portion of leinamycin (Table 2 and Figure 2). The cinnamylidenenacetophenones were evaluated for cytotoxicity against MDA-MB-468 and MCF-7 cells and the non-tumorigenic MCF-10A cells as seen in Table 3. When comparing **4** and **10**, **5** and **18,** and **6** and **9,** we detected a distinct decrease in cytotoxicity in MCF-10A cells when the conjugation was extended from the α,β–unsaturated ketone to the α, β–γ, δ–unsaturated ketone. Though extending the conjugation appeared to not appreciably impact breast cancer cell viability for the unsubstituted or trimethoxy substituted chalcone (**6** vs. **9 or 4** vs. **10**), such extension did improve *in vitro* cytotoxicity for both breast cancer cell lines for the 2-nitro-substituted chalcone (**5** vs. **18**).

With respect to the unsubstituted cinnamylideneacetophenone **9**, the substitution of trimethoxy phenyl for the unsubstituted phenyl as the A-ring (**10**) resulted in a substantial enhancement in cytotoxicity in both the MDA-MB-468 and the MCF-7 breast cancer cells. However, pronounced cytotoxicity was detected in MCF-10A cells following exposure to **10** as compared to **9**. Surprisingly, the presence of a single methoxy group in the 4- position on the A-ring seen in **11** resulted in complete loss of cytotoxic activity. The presence of a fluorine group in the 4- position on the A-ring as seen in **12** resulted in a loss of cytotoxicity in MDA-MB-468 cells yet substantially increased cytotoxicity in MCF-7 cells as compared to **9**. Unlike **10**, fluorine substitution in the 4-position on the A-ring in **12** resulted in no toxicity in the MCF-10A cells. The presence of chlorine in the 4-position on the A-ring resulted in a derivative (**13**) that was completely inactive. 3,4-dichloro substitution on the A-ring resulted in derivative (**14**) which demonstrated cytotoxicity in the MDA-MB-468 and MCF-7 breast cancer cells although substantial cytotoxicity was also detected in the MCF-10A cells. Substitution of a nitro group in the 4-position of the A ring resulted in an inactive derivative **15** while insertion of a napthyl group in the A-ring resulted in derivative **16** with activity in MDA-MB-468 cells but no activity in MCF-7 or MCF-10A cells.

Since chalcone **5** exhibited some activity in MCF-7 cells with an IC_{50} of 3.75 μM though inactive in MDA-MB-468 cells, we synthesized two cinnamylideneacetophenones with a 2-nitro group on the B ring. We found that derivative **17** exhibited potent nanomolar activity in both the MDA-MB-468 and MCF-7 breast cancer cell lines though cytotoxicity was apparent in MCF-10A cells unlike **9**. Derivative **18** exhibited low micromolar activity in MDA-MB-468 cells and extremely potent activity in the nanomolar range in MCF-7 cells. In addition, there was no appreciable cytotoxicity detected in MCF-10A cells following exposure to **18**. None of the cinnamylideneacetophenones were as cytotoxic to the MCF-10A cells as the reference chemotherapeutic doxorubicin. Additionally, **18** displayed more potent anticancer activity in MCF-7 cells than doxorubicin (Table 3).

One key assessment into the suitability of a potential agent for the treatment of breast cancer is how selective the agent is in exhibiting cytotoxicity. Ideally, an agent will exhibit cytotoxicity only in malignant cells while sparing non-tumorigenic cells. We evaluated the extent of selective cytotoxicity for each of the compounds as well as doxorubicin by dividing the IC_{50} for the non-tumorigenic MCF-10A cells by the IC_{50} values determined for both breast cancer cell lines (Table 4). Among the chalcones, **5** showed 2.7-fold greater toxicity in MCF-7 cells as compared to the MCF-10A cells. Otherwise, the chalcones were actually more cytotoxic in MCF-10A cells than the breast cancer cells. This finding strengthened our rationale for synthesizing derivatives with extended conjugation as opposed to additional chalcones. We detected a greater than 11-fold selectivity for MCF-7 cells following exposure to **12**. We found nearly 6-fold selectivity of **16** for MDA-MB-468 cells. Thus, fluorine substitution on the phenyl group (ring A) and insertion of a phenyl group for a naphthyl group in ring A led to derivatives with promising activity and selectivity for MCF-7 and MDA-MB-468 cells respectively.

The selectivity indices for **17** in MDA-MB-468 and MCF-7 cells were 10- and 128-fold respectively. The greatest extent of selective cytotoxicity was found when MDA-MB-468 and MCF-7 cells were exposed to **18**; a derivative that is more than 35 times more selective

for MDA-MB-468 cells and more than 25,000 times more selective for MCF-7 cells. The micromolar and sub-micromolar activity in the cancerous cell lines along with the high SI of derivative **18** make it the most promising candidate of all the cinnamylideneacetophenones for future studies and derivation. The next most promising derivative **17** only differs from **18** in that ring A consists of a furan heterocycle rather than a phenyl group.

In conclusion, the majority of the derivatives bore substitutions on the A-ring yet the two most active derivatives bore a 2-nitro group on the B-ring. Both of these agents exhibited anticancer activity in the nanomolar to sub-micromolar range yet exhibited substantially less cytotoxicity in the MCF- 10A cells. In fact the selectivity index for both **17** and **18** appears to be superior to that observed with the established chemotherapeutic agent doxorubicin. Studies are underway to further develop **17** and **18** as agents to treat breast cancer.

Acknowledgments

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19. *In vitro* anticancer activity determination. The cytotoxic activity of the cinnamylideneacetophenone derivatives was determined using the Alamar Blue™ assay in accordance with a previously published method.[18]. Stock solutions of the derivatives were prepared in DMSO at a concentration of 100 mM. In this study we used MCF-7 (estrogen receptor positive and MDA-MB-468 (estrogen receptor negative) breast cancer cells in comparison to the non-tumorigenic MCF-10A cells. Briefly, cells were plated at their appropriate densities in 96 well plates and allowed to attach for 24 h before being exposed to varying concentrations of the derivatives for 72 h at 37°C. The Alamar Blue™ dye was then added to each of the wells for to initiate 4 h incubation. The plates were then placed in a fluorescence microplate reader (BioTek FLx 800) to measure fluorescence levels and the IC_{50} values determined using GraphPad Prism 5.0.

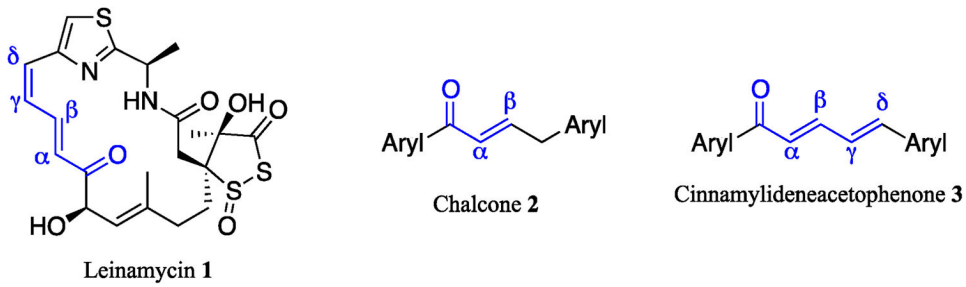


Figure 1.
The development of cinnamylideneacetophenones from common features of leinamycin and chalcone skeleton.

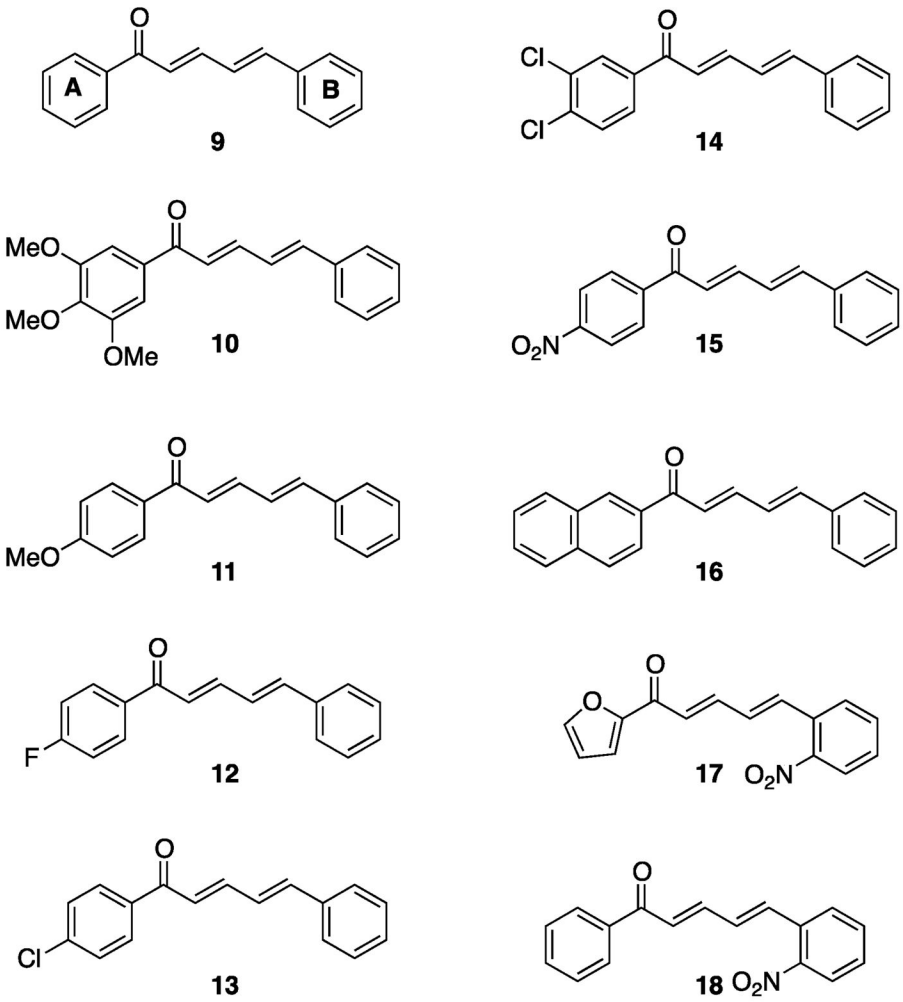
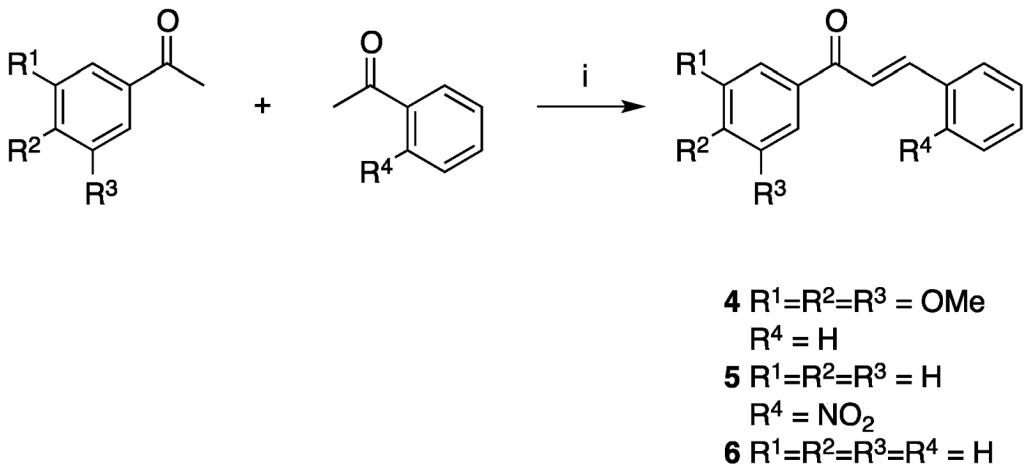
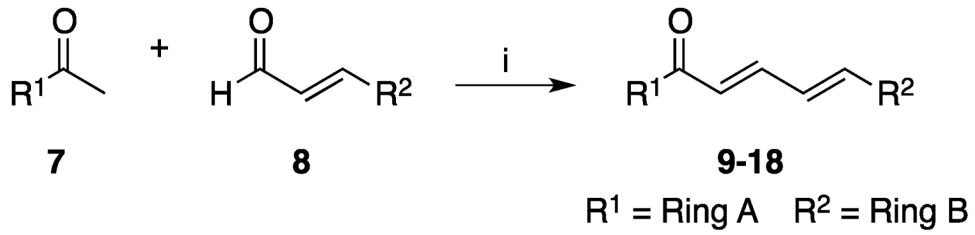


Figure 2.
The library of cinnamylideneacetophenones synthesized for biological evaluation.



Scheme 1.

Synthesis of chalcone derivatives **4** and **5**. (i) NaOH, H_2O/EtOH (50% v/v), rt, 24 h, 74–82%.



Scheme 2.
Construction of target molecules **9–18**. (i) NaOH, H_2O/EtOH (50% v/v), rt, 24h, 65–83%.

Table 1

Biological activity of chalcones **4–6** produced via Scheme 1[a]

Compound number	MDA-MB-468 IC_{50} (μM)	MCF-7 IC_{50} (μM)	MCF-10A IC_{50} (μM)
4	2.05 (1.27–3.32)	4.8 (1.99–11.8)	0.032 (0.004–0.28)
5	> 50	3.75 (1.17–11.9)	4.2 (0.9–17.8)
6	>50	> 50	3.15 (1.63–6.07)

[a] All tests were performed in replicate (n = 5) and repeated at least twice. Numbers in parentheses represent 95% confidence intervals.

Table 2

Cinnamylideneacetophenones **9–18** produced via Scheme 2[a]

Compound number	R^1	R^2
9	Ph	Ph
10	3,4,5-trimethoxyphenyl	Ph
11	4-methoxyphenyl	Ph
12	4-fluorophenyl	Ph
13	4-chlorophenyl	Ph
14	3,4-dichlorophenyl	Ph
15	4-nitrophenyl	Ph
16	Naphthyl	Ph
17	Furyl	2-nitrophenyl
18	Ph	2-nitrophenyl

Table 3

Cinnamylideneacetophenones **9–18** produced via Scheme 2[a]

Compound number	MDA-MB-468 IC_{50} (μM)	MCF-7 IC_{50} (μM)	MCF-10A IC_{50} (μM)
9	31.9 (13.4–75.7)	>50	>50
10	6.35 (1.4–10.4)	2.13(0.24–18.4)	1.11(0.47–2.65)
11	>50	>50	>50
12	>50	4.28 (0.13–1.4)	>50
13	>50	>50	>50
14	15.6 (6.7–36.3)	2.7 (0.5–14.6)	1.37 (0.257–7.5)
15	>50	>50	>50
16	8.429 (4.2–16.9)	>50	>50
17	0.78 (0.48–1.4)	0.071 (0.027–0.18)	9.11(1.5–56.4)
18	1.37 (0.74–2.5)	0.0019 (0.0005–0.0076)	>50
doxorubicin	0.1 (0.015–0.67)	0.18 (0.06–0.53)	0.037 (0.026–0.54)

[a] All tests were performed in replicate (n = 5). Numbers in parentheses represent 95% confidence intervals.

Table 4

Selectivity Index (SI)[b] for doxorubicin, chalcones and cinnamylideneacetophenones

Compound number	MDA-MB-468 SI	MCF-7 SI
4	0.016	0.004
5	<0.20	2.7
6	<0.096	<0.063
9	>1.6	N/A
10	0.17	0.17
11	N/A	N/A
12	N/A	>11.9
13	N/A	N/A
14	0.088	0.5
15	N/A	N/A
16	>5.95	N/A
17	10.24	128.3
18	>36.50	>26,316
doxorubicin	0.37	0.21

[b] SI = IC_{50} for MCF-10A cells/IC_{50} for cancer cells

N/A = not available