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Design and Synthesis of Indolyl Chalcone Analogues and Evaluation of Their Osteogenic Activity

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Abstract

We have designed and synthesized a series of novel functionalized indolyl chalcones and flavone conjugates *via* Claisen-Schmidt reaction. All the synthesized compounds were evaluated for their osteogenic activity. Among the synthesized compounds, four compounds showed significantly increased ALP activity whereas the novel compound **3b** has shown significant bone matrix mineralization and mRNA expressions of osteogenic marker genes, BMP2, RUNX-2 and OCN at 1 pM concentration.

Keywords: Chalcone derivatives; Claisen-Schmidt reaction; Indolyl chalcones; Osteoporosis; ALP activity, Bone matrix mineralization

Introduction

Osteoporosis is defined as generalized skeletal disorder characterized by compromised bone strength and deterioration of bone quality. Bone fragility fractures are the hallmark of osteoporosis and are particularly common in the spine, hip and forearm but may also affect other sites [1]. The pathogenesis of osteoporosis is multifactorial, including low peak bone mass, hormonal factors, use of certain drugs (e.g., glucocorticoids), cigarette smoking, low physical activity, low intake of calcium and vitamin D, race, small body size, post menopause and a personal or a family history of fracture. Osteoporosis can occur in both men and women and at any age, but it is most common in older women. Approximately, 1 in 3 women over age 50 will experiences osteoporosis, as well as 1 in 5 men aged over 50 annually [2]. Overall, 61% of osteoporotic problems occur in women, with female-to-male ratio of 1.6 [3]. Clinically, bone strength is estimated by non-invasive assessment of bone mineral density (BMD) by dual-energy X-ray absorptiometry (DXA).

Currently, the treatment options for osteoporosis include anti-resorptive agents like bisphosphonates (alendronate and zoledronate) and SERMs like raloxifene. These agents though prevent bone loss but does not increase bone mass [4]. Other treatment modalities include agents like Denosumab, which is a human monoclonal antibody against receptor activator for NFKB ligand (RANKL) critical for osteoclastogenesis [5] and monoclonal antibody like sclerostin, a Wnt signaling inhibitor [6].

Teriparatide (PTH 1-34) is the only FDA approved bone anabolic drug that suppresses the bone resorption and also prevents fracture. However, PTH therapy is restricted to two years, because FDA has issued a red flag against PTH due to risk of osteosarcoma. Moreover, PTH is an expensive therapeutic option. Teriparatide is hence the last line of therapy for osteoporosis and is given in very severe cases [7]. Hence, the increasing burden of osteoporosis urgently requires effective preventive strategies aimed to maximizing peak bone density, preventing excessive bone loss and also promoting gain of bone mass. Thus our efforts are to discover new potential agents that promote osteoblast differentiation and may have a role in management of bone related disorder.

The chemistry of chalcones has generated intensive scientific studies throughout the world. Chalcone is an α, β-unsaturated aromatic ketone which shapes the central core for a variety of important biological activities [8]. Chalcones are contrasting natural products in edible and medicinal plants having beneficial effects for human health to protect from life threatening diseases [9]. They are important as structural motifs among biologically active molecules and also for combinatorial assembly of heterocyclic scaffolds.

Different chalcone analogues have been synthesized for their enormous biological activities such as antimicrobial [10],

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antimalarial [11,12], anticancer [13-15], anti-inflammatory [16,17], anti-leishmanial [18,19], antiprotozoal [20], anti-HIV [21], anti-oxidant [22], anti-obesity [23]. Very few chalcone analogues have been reported for its anti-osteogenic activity [24]. Similarly, flavones synthesized from chalcones have also been found to show promising activities such as anti-oxidant [25], anticancer [26], anti-inflammatory [27], antimalarial [28], antiprolifrative [29], antidiabetic [30] activities. Due to these phenomenal bioactivities of chalcones and flavones much more effort has been steadfast to the synthesis of chalcone and flavone analogues.

Indoles have received great attention not only in organic chemistry but also in medicinal chemistry due to their numerous biological activities. The anti-cancer drug molecules such as vincristine, vinblastine, vinorelbine, vindesine, mitraphylline are containing indole in their core of the skeleton. It is also found in different antihypertensive drugs such as vincamine, reserpine, perindopril and also trandolapril, amedalin, pindolol, siramesine as antidepressant, apaziquone as a antimicrobial, oxipertine and roxindole as antipsychotic and schizophrenia renders valuable biologically activity due to the presence of indoleheterocycle. Moreover a wide range of drugs for asthama, viral diseases, HIV, and sexual dysfunction containing also possess indole in their core structure. Several hybrid molecules have been synthesized by incorporation of indole with other pharmacophores and evaluated for different biological activities [31].

As per our endeavor for discovery of natural product inspired drugs for anti-osteoporotic activity, we have isolated and/or synthesized different nature inspired compounds for the management of osteoporosis [32-37]. Our research work was focused towards the development of novel chalcone indole hybrid analogues as therapeutic agents for anti-osteoporotic activity. Herein, we have reported the synthesis and anti-osteoporotic activity of novel indoly chalcone hybrids and also introduced the indole as heterocycle in the B-ring of flavones.

Results and Discussion

Chemistry

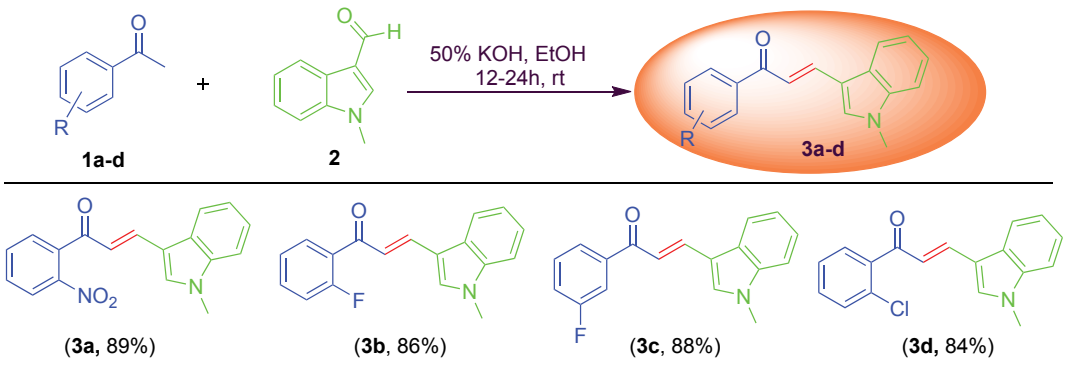
In order to achieve our drug discovery oriented venture, we designed and synthesized the hybrids of indole and chalcones by using Claisen-Schmidt reaction (Scheme 1 and 2). For the synthesis of these hybrid indolyl chalcones, we followed a synthetic route by taking N-methyl indole-3-carboxaldehyde and different acetophenones as starting materials. Initially, we were interested in the synthesis of indolyl chalcones without ortho-hydroxyl group.

Hence, the reaction mixture of substituted acetophenones (**1a-d**, 1.0 eq.), *N*-methyl indole-3-carboxaldehyde (**2**, 1.0 eq.) and 50% KOH (aq.) solution in ethanol (10 mL) was stirred at room temperature for 12 h to 24 h to get corresponding indolyl chalcone analogues (**3a-d**) in good yield (Scheme 1).

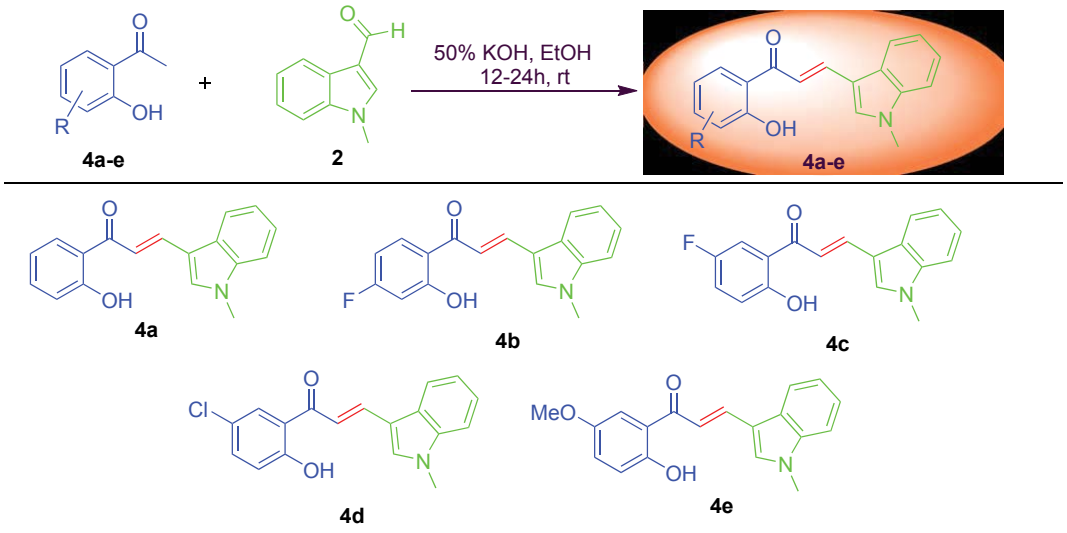
As several naturally occurring flavone analogues have been reported for their anti-osteoporotic activity and the flavones could be easily synthesized by their precursor ortho-hydroxyl group containing chalcones. Therefore, it is very important to evaluate the anti-osteoporotic potential of these precursor chalcones. As per our aim to the synthesis of indolyl chalcone hybrids, we carried out the reaction between 2′-hydroxyl acetophenones (**4a-e**, 1.0 eq.) and *N*-methyl indole-3-carboxaldehyde (**2**, 1.0 eq.) under Claisen-Schimdt reaction conditions to afford their respective indolyl chalcones (**4a-e**) in good yield (Scheme 2).

Now, the indolyl chalcones (**4a-d**) containing 2-hydroxyl group were further treated with 30% H_2O_2 (2.2 eq.) and 4N NaOH (2.0 eq.) in ethanol (7 mL) at 4°C-rt for 4 h to 6 h to afford corresponding indolyl flavones (**5a-d**) (Scheme 3). The details of the synthesis of all are mentioned in supporting information.

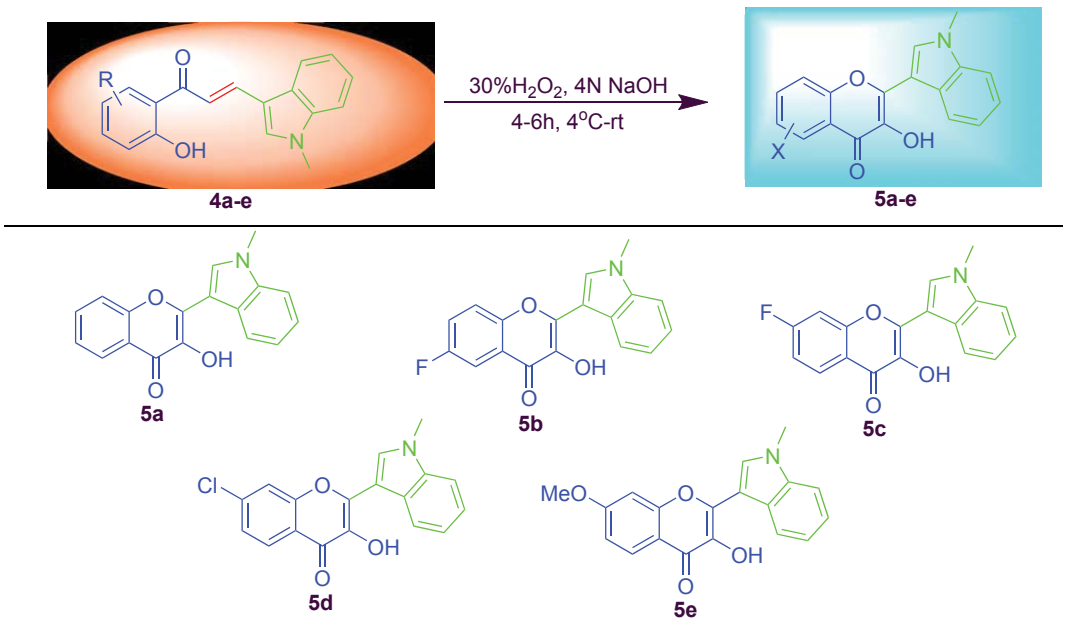
The structure of all synthesized compounds was established with the help of the analysis of FTIR, 1D & 2D NMR and mass spectrometric analysis. The purity of the synthesized compounds was analyzed by HPLC before bio-evaluation.



Scheme 1: Synthesis of indolyl chalcone hybrids having no ortho-hydroxyl group.



Scheme 2: Synthesis of indolyl chalcone hybrids having ortho-hydroxyl group.



Scheme 3: Synthesis of indolyl flavones.

Biological Activity

To determine the osteogenic activity of the synthesized compounds, osteoblast differentiation assay was performed *in vitro* because the osteoblast differentiation is one of the important aspects in osteoblast development. The alkaline phosphatase (ALP), osteocalcin (OCN) and type-1 collagen are some important bone markers which are frequently used to follow the differentiation process of osteoblasts. Thus, we decided to determine the osteogenic efficacy of compounds by measuring their ALP activity in mice calvarial osteoblasts. For measuring the alkaline phosphatase (ALP) activity, 2×10^3 cells per well were seeded in 96 well plates. Cells were treated with different concentrations of compounds for 48 h in α-MEM media containing 5% FCS, 10 mM *β*-glycerophosphate, 50 µg/ml of ascorbic acid, and 1% penicillin/streptomycin (osteoblast differentiation medium). After 48 h incubation, total ALP activity was measured using *p*-nitrophenyl phosphate (PNPP) as a substrate. Quantification was done colorimetric analysis at 405 nm.

Compounds **3a**, **3b**, **3c**, and **4b**, showed significant increase in ALP activity compared to the control untreated cells (Figure 1). Among the compounds under study, compound **3b** showed maximum osteogenic efficacy as assessed by ALP activity. It led to 100% and 85% enhancement in ALP activity at concentrations of 1 pM and 100 pM, respectively, which was the best amongst all other active compounds. Hence, **3b**

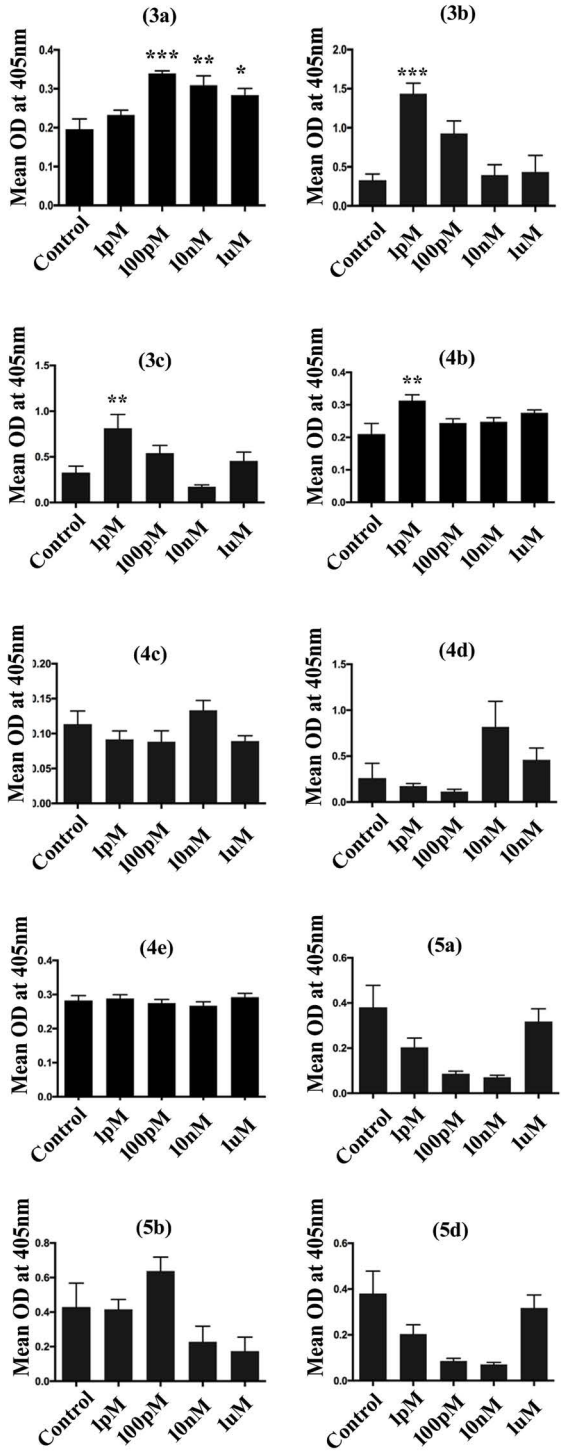


Figure 1: Effect of compounds on osteoblast differentiation. Mice calvarial osteoblasts (2 × 10^3 cells) were seeded in 96-well plate and treated with different concentrations of compounds for 48 h. ALP activity was quantified spectrophotometrically at 405 nm. Data shown as mean ± SEM; n=4; *P<0.05, **P<0.01 and ***P<0.001 compared with vehicle treated cells.

was further chosen for mineralization and qPCR experiments which determine the mRNA expression of osteogenic gene markers.

The process of osteoblastogenesis involves differentiation of preosteoblastic cells into terminally differentiated osteoblast cells mainly in three stages. These are proliferation, matrix development, maturation and mineralization. Mineralization is the most reliable indicator of differentiation of osteoblastic precursor cells to the terminal osteoblastic phenotype [38]. Therefore, osteogenic activity of compound **3b** was further evaluated in osteoblast mineralization assay. To assess the osteoblast mineralization efficacy of the compound, calvarial osteoblast cells were cultured for 18 days in differentiation media containing 10 mM *β*-glycerophosphate and 50 µg/ml ascorbic acid in presence of compound at 1 pM and 100 pM concentrations. Cells were then stained with alizarin red-S and dye was extracted to quantify the extent of osteoblast mineralization. The alizarin dye stains bind with newly formed mineralized calcium nodules. Optical densitometry was used to quantitate mineralization of treated osteoblast cells by measuring alizarin extracted from stained cultures. A significant increase exhibited by **3b** in mineral nodule formation at concentrations as low as 100 pM and 1 pM compared to the control untreated cells with best effect at 1 pM concentration (Figure 2A and 2B).

Bone morphogenetic proteins (BMPs) are members of transforming growth factor-β (TGF-β) super family. These

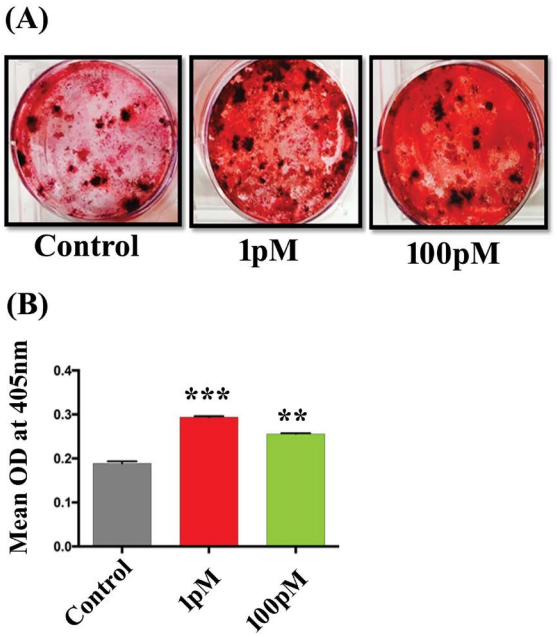


Figure 2: Effect of compound **3b** on mineralization of mice calvarial osteoblasts. 2 × 10^3 osteoblast cells were seeded in 12-well plates and exposed to different concentrations of compounds for 18 days. At the end of the incubation, cells were fixed and stained with alizarin red-S. Stain was extracted and O.D. measured colorimetrically as described in material and method. (A) Representative images of bone mineral nodule formation (B) Quantitative estimation of alizarin Red-S staining of mineralized nodules. Values are obtained from three independent experiments in the replicate of six/treatment point and expressed as mean ± SEM; **P<0.01 and ***P<0.01 compared with vehicle.

play an important role in osteoblast differentiation and bone regeneration. Among other BMPs, BMP-2 stimulates osteoblast phenotype expression such as increase in ALP activity and collagen synthesis [39]. Additionally, Runt-related transcription factor-2 (RUNX-2) is a protein which is a transcription factor associated with osteoblast differentiation and skeletal morphogenesis [40]. Further, osteocalcin (OCN) is an established differentiation marker for bone formation. Considering these markers as important indicators of bone formation, the mRNA expression levels of these markers were evaluated in presence of **3b** for further confirmation of its bone forming potential. To determine the effect of **3b** on osteogenic markers BMP-2, RUNX-2 and OCN, osteoblast cells were treated with compounds at 1 pM concentration for 24 h and 48 h, total RNA was isolated and cDNA was synthesized (Table 1). cDNA was used as a template in real time quantitative PCR. The house keeping gene GAPDH was used as the internal control in our experiments. Results showed that treatment of osteoblast cells with **3b** at 1 pM concentration increased the transcript levels of Runx-2 (~2.5 fold at 24 h), BMP-2 (~2.1 fold at 24 h) and OCN (~1.8 fold at 24 h), Runx-2 (~1.5 fold at 48 h), BMP-2 (~5.6 fold at 48 h) and OCN (~2 fold at 48 h) (Figure 3). Thus, compound **3b** up regulated the expression of osteogenic expression markers.

Gene Name	Primer Sequence
RUNX-2	F-TTGACCTTTGTCCCAATGC
	R-AGGTTGGAGGCACACATAGG
Osteocalcin	F-GCCCTGAGTCTGACAAAGGTA
	R-GGTGATGGCCAAGACTAAGG
BMP2	F-AGATCTGTACCGCAGGCACT
	R-GTTCCTCCACGGCTTCTTC
GAPDH	F-AGCTTGTCATCAACGGGAAG
	R-TTTGATGTTAGTGGGGTCTCG

Table 1: Primer sequences used for qPCR.

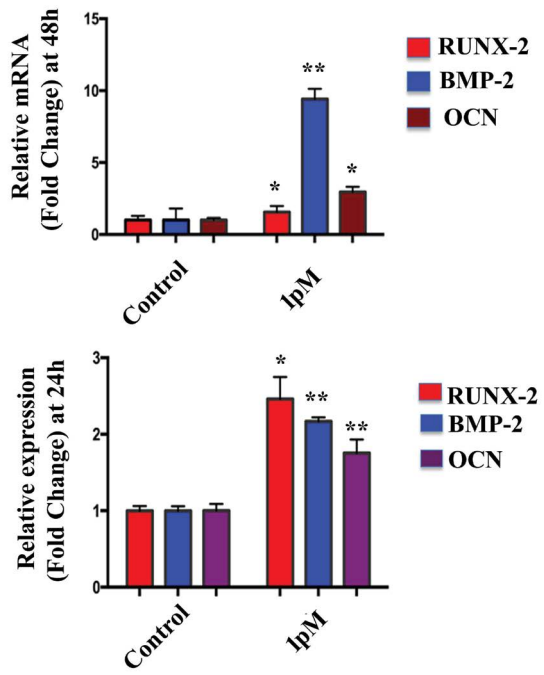


Figure 3: Effect of compound **3b** on mRNA expression of osteogenic markers. Osteoblast cells were cultured with or without compound **3b** and RNA was collected at 24 h and 48 h time point. qPCR for BMP-2, RUNX-2 and OCN was performed as described in materials and methods. Data shown as mean ± SEM; n=4; *P<0.05 and **P<0.01 compared with vehicle treated cells.

The structure activity relationship revealed that the indolyl chalcones without ortho-hydroxyl group showed significant osteogenic activity than indolyl chalcones with ortho-hydroxyl group and also its cyclized form i.e., indolyl-flavones. The compounds having ortho-fluoro instead of ortho-hydroxyl group showed more osteogenic activity. This is indicated that fluorine may be better substitute for hydroxyl group for designing of anti-osteoporotic agents. Inactive indolyl-chalcones indicated that the necessity of aryls instead of heteroaryls at C-2.

Conclusion

In conclusion, our study has shown the role of indolyl-chalcone and their cyclized compounds in osteoblast functions by stimulating mRNA expression of RUNX2, BMP2 and OCN bone morphogenetic proteins. We have also explored fluorine as a substitute of hydroxyl group for the designing of osteogenic agents. We have synthesized all these indole analogues by the application of Claisen-Schmidt reaction. Our preliminary results could be useful for the designing of novel heteroaryl and fluorine containing osteogenic lead molecules. Furthermore, the studies in search of more potent compound from these hybrid compounds and understanding of their mechanism of action are in progress.

Supplementary Material

Supplementary material is associated with this manuscript. It contains general experimental procedures, compound characterization data, and copies of ^{1}H and ^{13}C-NMR spectra of representative compounds.

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