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Novel indole α-methylene-γ-lactones as potent inhibitors for AKT-mTOR signaling pathway kinases

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Abstract—In an effort to generate novel anticancer agents, a series of hybrids of α-methylene-γ-lactones and 2-phenyl indoles has been synthesized and evaluated for inhibition activities on the phosphorylation of AKT, mTOR, p70S6 kinase, and 4E-BP1. The results indicate that substitutes on the γ-position of lactones have a rather significant influence on inhibition activities.

In recent years, great efforts have been made to improve the selectivity of anti-cancer agents by targeting the cancer-specific proteins or signaling pathways. Some small molecule compounds, such as gleevec,[1] against these targets have been developed and approved for use in clinical practice. On the other hand, these successes also reversely promote further studies on these new targets.

AKT-mTOR signaling pathway kinases, important components of intracellular signal systems, have been receiving increasing interest for their significant effect upon cell growth and oncogenesis. Mammalian target of rapamycin (mTOR) is considered a member of the PIK-related kinase family, with its C-terminus sharing strong homology to the catalytic domain of PI3-kinase (PI3K). PI3K and potentially protein kinase B (PKB; Akt) lie upstream of mTOR acting as apoptotic regulators, which are activated in many cancers and may promote drug resistance in vitro.[2] Ribosomal p70S6 kinase (p70S6K) and eukaryotic initiation factor-4E (eIF-4E) binding protein isoforms (4EBP1-3) are typical downstream mTOR effector molecules.[3] mTOR activates p70S6K by phosphorylating the Thr 389 of p70S6K. The p70S6K is recognized as the regulation kinase of the multiple phosphorylation of 40S ribosomal protein S6 in vivo so as to control the transcription of a class of mRNAs that contain an oligopyrimidine tract at the transcriptional start site.[4] eIF4E is negatively regulated by 4E-BP1. mTOR-mediated phosphorylation of 4E-BP1 dissociated the 4E-4E-BP1 complex, freeing 4E for its primary function of binding to the cap structure of mRNA as part of the translation initiation complex.[5] eIF4E exerts a mitogenic and oncogenic effect through activation of the Ras signaling pathway.[6]

Wortmannin (Fig. 1), isolated from the culture broth of *Talaromyces wortmannii* KY12420, has shown strong inhibitory activities for mTOR ($IC_{50} = 40$ nm).[7] Its activity may come from a near planar structure and an electrophilic exocyclic double bond conjugated to the carbonyl group of the δ-lactone ring.[8] Furthermore, indole rings, especially those substituted by alkyloxy or

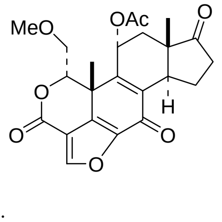

Figure 1. Wortmannin.

Keywords: Indole α-methylene-γ-lactones; AKT-mTOR signaling pathway kinase; Inhibitors.

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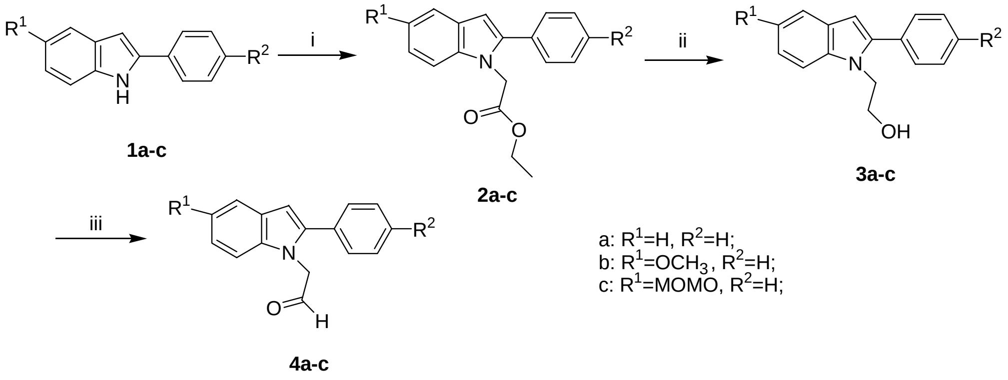
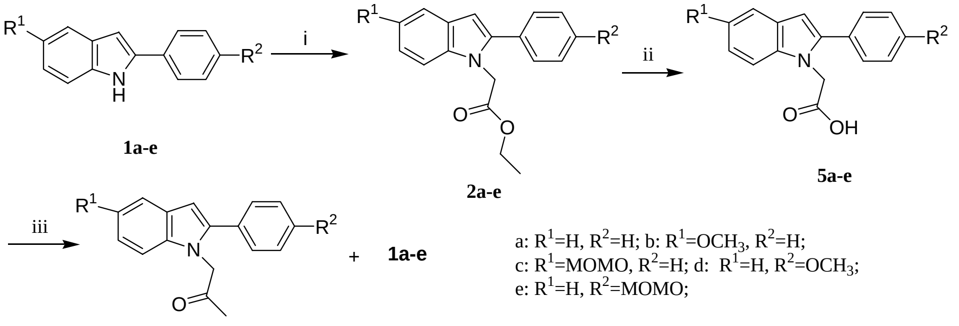


a: R^1=H, R^2=H;
b: R^1=OCH_3, R^2=H;
c: R^1=MOMO, R^2=H;

i: $BrCH_2COOEt$, NaH, DMF, rt., 8h; ii: $LiAlH_4$, THF, rt., 3h;
iii: DCC, Py, TFA, DMSO,Benzene, rt., 30h

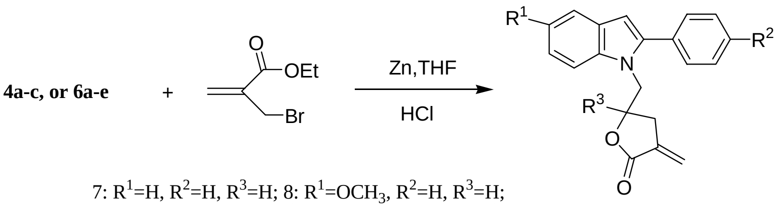
Scheme 1.



a: R^1=H, R^2=H; b: R^1=OCH_3, R^2=H;
c: R^1=MOMO, R^2=H; d: R^1=H, R^2=OCH_3;
e: R^1=H, R^2=MOMO;

i: $BrCH_2COOEt$, NaH, DMF, rt., 8h;
ii: NaOH, EtOH, 80 °C, 30min; iii: CH_3Li, Et_2O,rt., 2h;

Scheme 2.

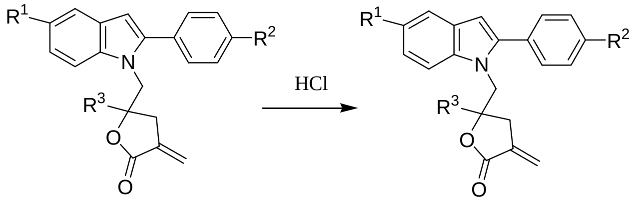


7: R^1=H, R^2=H, R^3=H; 8: R^1=OCH_3, R^2=H, R^3=H;
9: R^1=MOMO, R^2=H, R^3=H; 10: R^1=H, R^2=H, R^3=CH_3;
11: R^1=OCH_3, R^2=H, R^3=CH_3; 12: R^1=MOMO, R^2=H, R^3=CH_3;
13: R^1=H, R^2=OCH_3, R^3=CH_3; 14: R^1=H, R^2=MOMO, R^3=CH_3

Scheme 3.

hydroxy groups, are found in many natural products with anticancer activities.[9] Here, we report the preparation and biological evaluation of a series of novel conjugates that combine indoles and α-methylene-γ-lactones keeping functional groups of the δ-lactone ring, tending to find some small molecule compounds showing inhibitive activities to the AKT-mTOR signaling pathway kinases.

Ethyl 2-(bromomethyl)acrylate was prepared as reported in the literature,[10,11] and the indoles were obtained by the Fischer method. Schemes 1 and 2 illustrate the



15: R^1=HO, R^2=H, R^3=CH_3;
16: R^1=H, R^2=HO, R^3=CH_3.

Scheme 4.

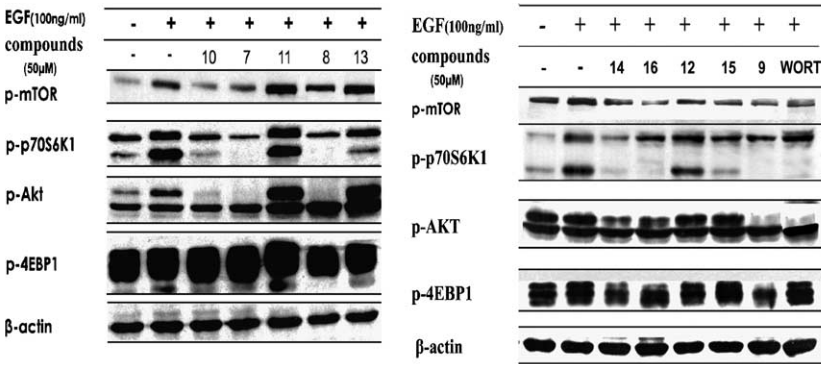


Figure 2. Western blot analysis was performed as follows. Rh30 cells were starved in serum-free RPMI-1640 culture medium for 24 h and then treated with 50 μM compounds for 1.5 h at 37 °C. Kinase phosphorylation was stimulated by 100 ng/ml EGF for 10 min. Cells (10^6) were collected by centrifugation at 3000 rpm for 3 min, washed with pre-cooled PBS for two times, and then resuspended in lysis buffer on ice for an hour. The lysates were centrifuged at 13,000 rpm for 15 min at 4 °C. Equivalent amounts of proteins were analyzed by 10% or 15% SDS–PAGE. After electrophoresis, the proteins were transferred onto nitrocellulose membrane (Millipore). After blocking the non-specific binding in blocking solution (5% non-fat milk in TBS/Tween 20), membranes were incubated with appropriate antibodies to: phosphorylated-AKT (1:1,000), phosphorylated-p70S6K (1:1,000), and phosphorylated-4EBP1 (1:1000), β-actin (1:1,000) at 4 °C overnight. Proteins were visualized with a peroxidase-coupled secondary antibody, using ECL-plus for detection. β-Actin served as loading control.

synthesis of *N*-indolyl-ketones or aldehydes **4** and **6**. We first used ethyl α-bromoacetate as the alkylation agent to obtain indole derivatives **2**. Compounds **2a–c** could then be reduced by $LiAlH_4$ to alcohols, which were subsequently oxidized to the corresponding aldehydes **4** via the Moffat reaction (Scheme 1). **2** could also be hydrolyzed to their corresponding carboxylic acids, and treated with methyl lithium, and then the ketones **6** were obtained with a lesser amount of indoles **2** (Scheme 2).

Interestingly, we found that when indole carboxylic acids were treated with alkyl lithium in dry Et_2O, the products were obtained readily. But when the reaction was carried out in a solution of dry THF, the corresponding indoles were obtained, while no ketones were produced. The desired compounds **7-14** were then synthesized by reacting intermediates **4** or **6** with ethyl 2-(bromomethyl)acrylate and zinc powder in dry THF (Reformatsky-type condensation) just as Öhler et al. had reported (Scheme 3).[12] Compounds **15** and **16** with a hydroxy substitute were prepared, respectively, by hydrolysis of the corresponding compounds **12** and **14** in dilute hydrochloride solution, as shown in Scheme 4.

The impact of compounds **7**–**16** on the phosphorylation of AKT, mTOR, p70S6K, and 4E-BP1 in RH30 cell line is shown in Figure 2. To exclude the interference of multiple growth factors in serum, we starved cells overnight and used 100 ng/ml EGF to stimulate unspecific reaction on many signal transduction kinases. Western blot results illustrated that four kinases we investigated were in a hypophosphorylated state after starvation and showed rather good identity when treated with target compounds.

In these 10 lactones, compounds **7**, **8**, and **9**, without possessing a methyl substituent at the γ-position of the lactone, showed more remarkable inhibitory effects on the phosphorylation of AKT, mTOR, p70S6 kinase, and 4E-BP1 at 50 μM than other methyl-containing compounds **10-16,** and the former compounds' activity is nearly equal to that of wortmannin in Figure 1. The good inhibition activity of compounds **7**, **8**, and **9** implies that less substitution on the lactones is favorable. However, compared to the apparent effect of space hindrance, the nature of substituents on indoles influences the inhibition ability slightly.

It can also be seen from Figure 1 that compounds **13**, **14**, and **16** with substituents on 2-phenyl ring show a better inhibition ability than their counterparts **11**, **12**, and **15** with substituents on the indole ring. Moreover, substitution with alkoxy group seems to be not advantageous as appearing in compounds **11**, **12**, and **13**.

The present work not only gives the chemical synthesis of novel α-methylene-γ-lactone-indoles hybrids but also their inhibition ability on the phosphorylation of AKT, mTOR, p70S6 kinase, and 4E-BP1. Initial results indicate that keeping the γ-position of the lactone not substituted is crucial for the inhibition activity. Besides, a methoxy substituent on the phenyl is more favorable than on the indole ring.

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