Synthesis and antitumor evaluation of neolaxiflorin B inspired compounds

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Background: Neolaxiflorin B is derived from ent-kaurane like laxiflorin J and eriocalyxin B with a relatively low potency as an antitumor agent. During preliminary structure–activity relationship studies, the α,β-unsaturated ketone (enone) system is an important active group.

Methods: Seven neolaxiflorin B derivatives containing α,β-unsaturated ketone moieties were synthesized. In vitro, activity was evaluated against three human tumor cell lines and a rat myogenic cell line (HepG2, NSCLC-H292, SNU-1040, and L6, respectively) by MTT assay.

Results: Compound 15 appeared a promising antitumor lead due to its cytotoxic potency and relatively high selectivity, with an SI value of 13.14. Flow cytometry analysis was conducted to show that NSCLC-H292 cells were blocked in the G0/G1 phase in the presence of compound 15, thus inhibiting the proliferation of tumor cells.

Conclusion: This study has revealed that compound 15 is a promising antitumor lead due to the cytotoxic potencies and the high selectivity it displayed when compared to natural counterparts.

Keywords: neolaxiflorin B inspired compounds, unsaturated ketone, antitumor activity

Introduction

In the past decade, over 1000 ent-kaurane diterpenoids have been isolated and identified from the Isodon genus.1 According to recent reports, these diterpenoids possess a broad range of bioactivities, including anti-bacteria,2 cytotoxic, anti-HIV, anti-inflammatory, and anti-fungal activity.3 Laxiflorin, as one of the members of the diterpenoids, is assumed to share the same biogenic precursor, ent-kaurene (1), with eriocalyxin B and longikaurin A (Figure 1).4 Biosynthetically, there is a possibility of mutual transformation among these diterpenoids.

According to the literature,5 neolaxiflorin B is derived from ent-kaurane like laxiflorin J and eriocalyxin B (Figure 2) with a relatively low potency as an antitumor agent. Although low in potency, its structure is novel, containing a unique scaffold, which we used as a basic template to synthesize novel natural product-like neolaxiflorin B derivatives to develop safe and effective antitumor agents.

During preliminary structure–activity relationship studies, the removal of the α, β-unsaturated ketone (enone) system in the D-ring decreased its antitumor activity.6 Meanwhile, mounting evidence also demonstrates that dieneone compounds with double α,β-unsaturated ketone functionalities, such as eriocalyxin B (3), can undergo two successive alkylations at the β-positions by reacting with cellular thiols during biological cascade reactions.7 We, therefore, aimed to synthesize a neolaxiflorin B-like scaffold with double α,β-unsaturated ketone.
CC was performed by using silica gel (100–200 mesh; Qingdao Marine Chemical, China)

4-(hydroxymethyl)-3-isopropylcyclopent-2-enone (6)
To a solution of 5 (0.248 g, 2 mmol, 1eq) in n-hexane (5 mL) at room temperature under argon was added Co$_2$(CO)$_6$ (0.684 g, 2 mmol, 1eq). After 1 hr, the mixture was filtered through a short pad of neutral Al$_2$O$_3$ washed with n-hexane. The filtrate was added neutral Al$_2$O$_3$ (10 g) and concentrated under vacuum. To remove traces of oxygen, the residue is kept at 0.08 MPa for 15 mins, then the flask is flushed with argon. The procedure is repeated four times, further heated for 2 hrs at 50°C. The desired product 6 (70%) was achieved by silica gel column chromatography as a pale yellow liquid: $^1$H NMR (300 MHz, CDCl$_3$) δ=5.83(s, 1H), 3.82–3.60 (m, 3H), 3.03 (s,1H), 2.58 (m,1H), 2.45–2.22 (m, 2H), 1.10 (dd, J=7.8 Hz, 6H) ppm; $^{13}$C NMR (75.5 MHz, CDCl$_3$) δ=210.11, 189.49, 128.58, 62.56, 44.40, 39.32, 29.57, 21.37, 20.44ppm; IR (KBr): umax =3388.32, 2964.75, 2874.24, 1718.07, 1676.32, 1603.50 cm$^{-1}$; MS (ESI): m/z: 291; HRMS (ESI) Calcd for ([C15H28O2Si]+Na): 291.1756; found: 291.1758[M+Na]$^+$.  

Compound 7
To a solution of 6 (0.38 g, 2.47 mmol, 1eq) and imidazole (0.34 g, 4.94 mmol, 2eq) in CH$_2$Cl$_2$ (15 mL) at room temperature was added tert-Butyldimethylsilyl chloride (0.56 g, 3.71 mmol, 1.5eq). The solution was stirred overnight, and then the mixture was added water and extracted with CH$_2$Cl$_2$. The combined organic layers were dried over Na$_2$SO$_4$ and the solvent was removed under reduced pressure. The residue was purified by flash column chromatography to provide 7 (96%) as colorless liquid: $^1$H NMR (300 MHz, CDCl$_3$) δ=5.93(s, 1H), 3.71(d, J=4.5 Hz,2H), 3.07(m, 1H), 2.66–2.62(m, 1H), 2.51–2.43(m, 1H), 2.30–2.23(m, 1H), 1.17(d, J=6.9 Hz, 3H), 1.14(d, J=7.2 Hz, 3H), 0.83(s, 9H), 0.01(s, 6H)ppm; $^{13}$C NMR (75.5 MHz, CDCl$_3$) δ=209.19, 188.59, 128.81, 63.50, 44.42, 39.51, 29.74, 25.83, 21.76, 20.74, 18.24, 18.11, –3.46, –5.48ppm; IR (KBr): umax =2959.33, 2928.81, 2874.91, 2857.09, 1718.07, 1675.32, 1603.50 cm$^{-1}$; MS (ESI): m/z: 291; HRMS (ESI) Calcd for ([C15H28O2Si]+Na)$^+$: 291.1756; found: 291.1758[M+Na]$^+$.  

Compound 8
Compound 8 is obtained as pale yellow liquid: $^1$H NMR (300 MHz, CDCl$_3$) δ=7.43(d, J=9.9 Hz, 1H), 5.81(d, J=9.9 Hz, 1H), 5.26(s, 1H), 5.05(s, 1H), 4.19(q, J=7.2 Hz, 2H), 3.47(br., —br.): 177.0888[M+Na]$^+$.
1H), 2.86(d, J=95.4 Hz, 1H), 2.52(d, J=95.4 Hz, 1H), 2.25(m, 2H), 1.25(t, J=7.2 Hz, 3H) ppm; 13C NMR (75.5 MHz, CDCl3) δ=197.515, 172.761, 152.359, 143.136, 126.641, 113.126, 61.584, 57.960, 51.617, 43.207, 41.109, 14.120 ppm; IR (KBr): umax =2979.98, 1729.06, 1685.40, 1280.90, 1222.04, 1193.09 cm⁻¹; MS (ESI): m/z: 497; HRMS (ESI) Calcd for ([C27H40O6Si]+Na)⁺: 513.2648; found: 513.2655 [M+Na]⁺.

**Compound 10**

Compound 10 is obtained as white powder; m.p.: 123–128°C; 1H NMR (400 MHz, CDCl3) δ=5.83(s, 1H), 5.07(s, 1H), 4.91 (s, 1H), 4.13(q, J=7.2 Hz, 2H), 3.86(dd, J1=10 Hz, J2=2.8 Hz, 1H), 3.71(dd, J1=10 Hz, J2=3.6 Hz, 1H), 3.23(s, 1H), 3.20(d, J=12 Hz, 1H), 2.79–2.68(m, 6H), 2.23(s, 1H), 2.26–2.21(m, 1H), 1.80(d, J=16.8 Hz, 1H), 1.24(t, J=7.2 Hz, 3H), 1.12(d, J=6.8 Hz, 3H), 1.07(d, J=6.8 Hz, 3H), 0.73(s, 1H), 0.78(s, 3H) ppm; 13C NMR (75.5 MHz, CDCl3) δ=197.515, 172.761, 152.359, 143.136, 126.641, 113.126, 61.584, 57.960, 51.617, 43.207, 41.109, 14.120 ppm; IR (KBr): umax =2979.98, 1729.06, 1685.40, 1280.90, 1222.04, 1193.09 cm⁻¹; MS (ESI): m/z: 497; HRMS (ESI) Calcd for ([C27H40O6Si]+Na)⁺: 513.2648; found: 513.2655 [M+Na]⁺.

**Compound 11**

To a solution of 9 (0.47 g, 1 mmol, 1 eq) in DCM, SeO2 (0.1 g, 0.9 mmol, 0.9 eq) was added in one portion, and a solution of t-BuOOH (0.55 mL, 3 mmol, 5.5 M in decane, 3 eq) was added dropwise. The solution was vigorously stirred at room temperature overnight, and then the mixture was filtered through a short pad of silica gel washed with EtOAc. The filtrate was concentrated under vacuum and purified by flash column chromatography to provide allylic alcohol 11 (71%) as colorless liquid: 1H NMR (400 MHz, CDCl3) δ=6.00(s, 1H), 5.42(s, 1H), 5.33(s, 1H), 4.61 (s, 1H), 4.26(q, J=8 Hz, 2H), 3.85(m, 1H), 3.72m, 1H), 3.34 (d, J=4 Hz, 1H), 3.09(d, J=8 Hz, 1H), 2.69–2.38(m, 4H), 2.15–2.02(m, 3H), 1.60(d, J=16 Hz, 1H), 1.28(t, J=8 Hz, 3H), 1.17(d, J=4 Hz, 3H), 1.13(d, J=8 Hz, 3H), 0.77(s, 9H), 0.00(s, 3H), –0.02(s, 3H) ppm; HRMS (ESI) Calcd for ([C27H40O6Si]+Na)⁺: 513.2648; found: 513.2655 [M+Na]⁺.
General procedure for deprotection of silyl ether
HClO$_4$·SiO$_2$ (0.5 mmol/g, 50 mg) was added to a stirred solution of silyl ether substrate (1 mmol) in CH$_2$Cl$_2$ (5 mL) at room temperature. After complete conversion and filtration to remove the solid, saturated aqueous solution of NaHCO$_3$ was added and separated. The aqueous solution was extracted with CH$_2$Cl$_2$ (5 mL×3). The organic layer was combined, washed with brine (5 mL), dried over MgSO$_4$, and concentrated under reduced pressure. The residue was isolated through short column chromatography on silica gel, which was eluted with ethyl acetate-petroleum to give the target products.

**Compound 13**
Compound 13 is obtained as colorless liquid: $^1$H NMR (400 MHz, CDCl$_3$) δ = 6.04(s, 1H), 5.08(s, 1H), 5.07(s, 1H), 4.22(q, $J$=5.76 Hz, 2H), 3.90(dd, $J_1$=2.96 Hz, $J_2$=8.88 Hz, 1H), 3.68(dd, $J_1$=4.64 Hz, $J_2$=8.88 Hz, 1H), 3.28(dd, $J_1$=4.12 Hz, 1H), 3.05–2.83(m, 4H), 2.66–2.05(m, 4H), 2.29–2.17(m, 3H), 1.29(t, $J$=5.76 Hz, 3H), 1.20(d, $J$=5.28 Hz, 3H), 1.18(d, $J$=5.64 Hz, 3H)ppm; $^{13}$C NMR (100 MHz, CDCl$_3$) δ = 207.98, 206.55, 199.67, 186.67, 169.01, 143.03, 129.05, 119.95, 63.77, 61.78, 61.07, 54.58, 51.33, 50.18, 41.52, 38.92, 32.79, 29.82, 21.47, 20.57, 14.04ppm; IR (KBr): $\nu$ = 3434.28, 3077.84, 2968.23, 2919.88, 2876.52, 1715.33, 1693.68, 1642.11, 1608.62, 1466.21 cm$^{-1}$; MS (ESI): m/z: 397; HRMS (ESI) Caled for [(C21H28O5)+Na]$^+$: 397.1627; found: 397.1629 [M+Na]$^+$.

**Compound 14**
Compound 14 is obtained as colorless liquid: $^1$H NMR (400 MHz, CDCl$_3$) δ = 6.08(s, 1H), 5.46(s, 1H), 5.36(s,1H), 4.66(s, 1H), 4.30(q, $J$=5.72 Hz, 2H), 4.12(q, $J$=5.72 Hz, 2H), 3.91(dd, $J_1$=2.96 Hz, $J_2$=8.88 Hz, 1H), 3.76(dd, $J_1$=4 Hz, $J_2$=8.84 Hz, 1H), 1.37(d, $J$=4.2 Hz, 1H), 3.11(d, $J_1$=7.4 Hz, 1H), 2.83–2.36(m, 5H), 2.15(m, 2H), 1.34(t, $J$=5.68 Hz, 3H), 1.21(d, $J$=5.32 Hz, 3H), 1.18(d, $J$=5.64 Hz, 3H)ppm; $^{13}$C NMR (100 MHz, CDCl$_3$) δ = 208.42, 207.70, 189.86, 173.27, 149.67, 129.13, 114.97, 78.91, 63.00, 61.72, 60.42, 57.00, 52.22, 47.11, 40.61, 35.56, 32.16, 29.79, 21.63, 20.59, 14.20ppm; IR (KBr): $\nu$ = 3434.70, 3080.00, 2967.55, 2933.39, 2877.04, 1716.14, 1608.85, 1467.55 cm$^{-1}$; MS (ESI): m/z: 399; HRMS (ESI) Caled for [(C21H28O6)+Na]$^+$: 399.1784; found: 399.1788 [M+Na]$^+$.

**Compound 15**
Compound 15 is obtained as colorless liquid: $^1$H NMR (400 MHz, CDCl$_3$) δ = 6.16(s, 1H), 5.98(s, 1H), 5.55(s, 1H), 4.20(q, $J$=5.72 Hz, 2H), 3.88(dd, $J_1$=3.2 Hz, $J_2$=8.52 Hz, 1H), 3.63(dd, $J_1$=5.04 Hz, $J_2$=8.52 Hz, 1H), 3.02–2.82(m, 3H), 2.63–2.38(m, 5H), 2.16(m, 2H), 1.30(t, $J$=5.72 Hz, 3H), 1.18(d, $J$=5.32 Hz, 3H), 1.16(d, $J$=5.64 Hz, 3H)ppm; $^{13}$C NMR (100 MHz, CDCl$_3$) δ = 207.98, 206.55, 199.67, 186.67, 169.01, 143.03, 129.05, 119.95, 63.77, 61.78, 61.07, 54.58, 51.33, 50.18, 41.52, 38.92, 32.79, 29.82, 21.47, 20.57, 14.04ppm; IR (KBr): $\nu$ = 3434.28, 3077.84, 2968.23, 2919.88, 2876.52, 1715.33, 1693.68, 1642.11, 1608.62, 1466.21 cm$^{-1}$; MS (ESI): m/z: 397; HRMS (ESI) Caled for [(C21H28O6)+Na]$^+$: 397.1627; found: 397.1629 [M+Na]$^+$.

**Cell culture and MTT test**
Cells were purchased from the Wuxi Innovatbio Medicine Technology Co. Ltd (Wuxi, China) and were maintained at 37°C under the atmosphere of 5% CO$_2$. The cells of human non-small lung cancer (H292) and colon cancer cell line (SNU-1040) cells were cultured in RPMI-medium and others (rat myoblasts and HepG2) were cultured in dulbecco’s modified eagle medium. Ten percent fetal bovine serum (Tianhang Biotechnology Co., Ltd., Zhejiang, China.) and 1% antibiotics (100 U/mL penicillin and 100 mg/mL streptomycin) was supplemented. Cells are inoculated in 96-well plate and cultured for 24 hrs. After attaching the culture bottle wall, the cells were divided into three groups, 1) control group; 2) positive control group; 3) experiment group. The administration concentrations of Cisplatin and compounds were 0.1 µM–10 µmol/L. After 24 hrs, 10% MTT was added and cells were cultured for 3.5 hrs. Then, the culture medium was discarded and 150 µL DMSO was added. Eventually, after incubation for 10 mins by shaking in the dark at room temperature, the absorbance was detected at 490 nm using a microplate reader, and the difference was analyzed by prism 7 software.

**Flow cytometry**
Cells were cultured in a six well plate and administrated with DTT and compound 15 for 48 hrs. After that, cells were digested and transferred into polyethylene pipe, followed by a centrifugation at 1000 for 5 mins. Then, 1 mL of precooled PBS was given to suspend the cells again. After another centrifugation, PBS was discarded and 70% ethanol was added to fix the cells overnight at 4°C. After rinsing with PBS, the cells were added to propidium iodide staining solution according to instructions. (Beyotime, China). The red fluorescence of the cells (1×105) was detected at 488 nm wavelength by flow cytometer (BD ACCURI C6 PLUS).
Results and discussion

We constructed dienone functionality in the A and D-ring and deleted the complex spiral B-ring moiety of neolaxiflorin B. Compound 6 was obtained through the Pauson-Khand reaction. Starting material 5 was treated with 10 mol% Chlorobis(ethylene) rhodium (I) dimer ([RhCl(COD)]$_2$) under CO at 1atm in toluene at 100°C, but the desired product was not observed. Treatment of 5 with Molybdenum hexacarbonyl (Mo(CO)$_6$) was also unsuccessful. We found that the conditions using classical dicobalt species Octacarbonyldicobalt (Co$_2$(CO)$_8$) for the Pauson-Khand reaction, however, were successful. We evaluated the Pauson-Khand reaction conditions for the synthesis of compound 6 (Table 1). The optimum conditions for 6 were achieved using the following procedure: Compound 5 was mixed with stoichiometric Co$_2$(CO)$_8$ in n-hexane under an inert argon atmosphere at rt. After 1 hr, neutral Al$_2$O$_3$ was added, and the solvent was removed using a rotary evaporator. The residue was incubated at 0.08 MPa for 15 mins at room temperature, then the flask was flushed with argon. The 0.08 MPa and argon flush steps were repeated four times and further heated at 50°C for 2 hrs. The desired product 6 was achieved by silica gel column chromatography.

A silylation reaction of compound 6 was used to produce compound 7. Deprotonation of compound 7 with Lithium bis(trimethylsilyl)amide (LiHMDS), followed by reaction with compound 8 led to product 9 as the major product, along with the isomer 10 in 86% combined yield (dr =9:1) (Scheme 1). Compound 9 is a crystal that was confirmed by NMR spectral and X-ray crystallographic analysis (Figure 3), its epimer compound 10 is a powder.

Compound 9 was subjected to allylic oxidation with selenium dioxide (SeO$_2$), tert-butyl hydroperoxide (t-BuOOH) in

**Table 1** Pauson–Khand reaction of compound 5 for the synthesis of compound 6

| Catalyst        | Solvent     | Temp. (°C) | %yield |
|-----------------|-------------|------------|--------|
| [RhCl(COD)]$_2$/CO | Toluene     | 100        | –      |
| Mo(CO)$_6$      | Toluene     | 100        | –      |
| Co$_2$(CO)$_8$  | Toluene     | 100        | –      |
| Co$_2$(CO)$_8$  | DMF         | 100        | –      |
| Co$_2$(CO)$_8$  | MeOH        | 50         | –      |
| Co$_2$(CO)$_8$  | CH$_2$Cl$_2$| rt         | –      |
| Co$_2$(CO)$_8$  | n-hexane    | rt         | trace  |
| Co$_2$(CO)$_8$  | n-hexane/SiO$_2$/Ar | 40     | 40     |
| Co$_2$(CO)$_8$  | n-hexane/Al$_2$O$_3$/Ar | rt-50 | 70     |

Scheme 1 Synthesis of compounds 7, 9, and 10.

Figure 3 X-ray crystallographic analysis of compound 9.
CH₂Cl₂, and 2-Iodoxybenzoic acid (IBX) oxidation to form compounds 11 and 12, respectively (Scheme 2). Deprotection of the silyl ethers of compounds 9, 11, and 12 formed alcohol products in the presence of perchloric acid (HClO₄) (Scheme 3).

The cytotoxic properties of all newly synthesized compounds were evaluated in vitro against three human tumor cell lines (HepG2, NSCLC-H292, SNU-1040) and one rat myotubes cell line (L6) by MTT assay. Eriocalyxin B (5) was used as reference drug. The results are summarized in Table 2. (IC₅₀ value is defined as the concentrations corresponding to 50% growth inhibition).

Results are displayed in Table 2. From the table, compounds 9 (IC₅₀ =1.01 μM, NSCLC-H292), 10

Table 2 In vitro cytotoxic activities of compounds 9–15 [IC₅₀, μM]ᵃᵇ

| Compounds | Cell lines | HepG2 | NSCLC-H292 | SNU-1040 | L6 |
|-----------|------------|-------|------------|----------|----|
| 9         |            | >30   | 1.01       | 2.97     | 37.60 |
| 10        |            | >30   | 3.67       | 5.25     | 17.55 |
| 11        |            | 1.58  | 5.73       | 7.08     | 48.58 |
| 12        |            | 2.00  | 1.08       | 2.85     | 80.60 |
| 13        |            | 3.48  | 3.48       | 1.23     | 27.61 |
| 14        |            | 3.95  | 3.05       | 1.08     | 23.98 |
| 15        |            | 1.76  | 1.66       | 3.17     | 216.93 |
| EriocalyxinB |         | 2.12  | 7.11       | 1.54     | 115.17 |

Notes: aCytotoxicity as IC₅₀ values for each cell line, the concentration of compound that caused 50% reduction in absorbance at 570 nm relative to untreated cells using the MTT assay. bHuman liver carcinoma cell line (HepG2), non-small cell lung cancer cell line (NSCLC-H292), human colon cancer cell line (SNU-1040), normal skeletal muscle of rat myotubes (L6).
(IC₅₀ =3.67 μM, NSCLC-H292), 11 (IC₅₀ =1.58 μM, HepG2), 13 (IC₅₀ =1.23 μM, SNU-1040), and 14 (IC₅₀ =1.08 μM, SNU-1040) showed potency when comparing to the reference eriocalyxin B with only an enone in the A-ring. Meanwhile, these results indicated that the presence of hydroxyl groups at the 6 and 15 position had no effect on the activity. Epimers 9 and 10 did not show significant differences in cytotoxicity and selectivity, so we chose compound 9 that was more readily available for further modification. Remarkably, compounds 12 and 15 with a diene in the A and C ring, showed stronger activity against all tumor cell lines compared to those with one enone (compounds 9, 10, 11, 13, and 14). Deprotection of C-6 silyl ether of compound 12 produced compound 15, which greatly decreased the cytotoxic activity against rat myoblast cell line (L6). To obtain insight into the cytotoxic potential of these new compounds on normal cells, the effect of compound 15 and eriocalyxin B was evaluated in human liver carcinoma cells (HepG2) and rat myotubes (L6) cells. The potency and selectivity of compound 15 were higher than that of eriocalyxin B. The SI value (selective index, IC₅₀ of normal cells/IC₅₀ of tumor cells) of compound 15 was 123.14, and the SI value of eriocalyxin B was 54.33.

After cytotoxic assessment experiment, we conducted flow cytometry analysis to further understand potential underlying cellular mechanisms of actions of compound 15 (Table 3).

We found that the percentage of NSCLC-H292 cells in G0/G1 phase increased significantly but those in G2/M decreased notably in the presence of DTT and compound 15, indicating that cells were blocked in G0/G1 phase, thus inhibiting the proliferation of tumor cells.

### Table 3 Percentage of G0/G1, S, and G2/M phase

| Groups | G0/G1 | S     | G2/M  |
|--------|-------|-------|-------|
| Control| 23.63±1.39 | 37.02±1.37 | 18.90±0.76 |
| DTT 10 μM | 46.07±0.86*** | 24.11±0.64 | 14.20±1.29 |
| DTT 1 μM | 44.32±0.87*** | 21.09±1.75 | 11.12±0.92* |
| DTT 0.1 μM | 40.67±0.44*** | 23.28±0.64 | 15.78±1.55 |
| 15 10 μM | 43.98±0.83***,# | 23.44±1.40 | 10.12±0.72* |
| 15 1 μM | 37.65±1.88***,### | 29.77±0.71 | 11.65±0.93* |
| 15 0.1 μM | 33.62±1.37,## | 31.9±0.49 | 13.09±0.56 |

Notes: Values are means ± S.E.M. Data were obtained from five separate experiments (n=5). Significance of the difference between groups was indicated as follows: *Compared with Control groups, # compound 15 compared with DTT. **P<0.01; ***P<0.001; ###P<0.001.

### Conclusion

In conclusion, we have designed and synthesized several compounds bearing an α, β-unsaturated ketone moiety. The in vitro activities were evaluated against HepG2, NSCLC-H292, and SNU-1040 tumor cell lines and an L6 myoblast cell line. This study has revealed that compound 15 is a promising antitumor lead due to the cytotoxic potencies and the high selectivity it displayed when compared to natural counterparts. Further biological investigations are currently undergoing to investigate how compound 15 inhibits tumor cell proliferation in our laboratory and the results will be reported in due course.

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### Disclosure

The authors report no conflicts of interest in this work.

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