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# The synthesis and anti-proliferative effects of β-elemene derivatives with mTOR inhibition activity

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**Abstract**—Fourteen β-elemene derivatives containing a piperazine, a morpholine, a tetrahydropyrrole, a thiophenylethylamine, or a cyclohexamine group were synthesized. The structures of these β-elemene derivatives were characterized with IR, $^1$H NMR, MS, and elemental analyses. All these derivatives had an increased anti-proliferative activity in human cervix epitheloid carcinoma HeLa, gastric carcinoma SGC-7901, and leukemia K562 cells comparing with that of β-elemene. Among these derivatives, 13,14-bis(*cis*-3,5-dimethyl-1-piperazinyl)-β-elemene (**IIi**), 13,14-bis[2-(2-thiophenyl)ethylamino]-β-elemene (**IIm**), and 13,14-bis(cyclohexamino)-β-elemene (**IIn**) were the most potent agents. **IIi**, **IIm**, and **IIn** inhibited K562 cell growth with an $IG_{50}$ below 5 μM that was correlated with mTOR activity inhibition.


## 1. Introduction

β-Elemene is one of the active components in the essential oil of *Curcuma* Wenyujin Y.H. Chen et C. Ling, a traditional Chinese herb medicine.[1] β-Elemene has been shown to inhibit tumor cell growth in vitro and in vivo, and has been put into clinical trials in cancer patients with observed efficacy.[2,3] However, due to a poor water solubility and a requirement of high concentrations to reach a therapeutic effect, the efficacy of β-elemene in cancer treatment is limited.

Structural modifications have been proved to successfully increase water solubility and/or anti-tumor activity of several natural compounds, such as that of camptothecin[4,5] and taxol.[6,7] It has been found that β-elemene derivatives containing a hydroxy moiety had an increased activity of inhibiting tumor cell growth comparing with that of β-elemene,[8] thus introduction of a hydrophilic group into the skeleton of β-elemene should increase its water solubility and tumor growth inhibitory effect. In this communication, a series of β-elemene derivatives containing a piperazine moiety or other amino groups were synthesized. Their activity of inhibiting tumor cell growth and the potential mechanisms were studied in a few of cancer cell lines.

## 2. Results and discussion

### 2.1. Synthesis

The synthetic pathway of fourteen compounds from β-elemene via chlorination and sequential N-alkylation is outlined in Scheme 1 and the substituted groups are listed.

### 2.2. Anti-proliferative effects in tumor cells

The growth inhibitory effects of compounds **IIa**–**IIn** in HeLa and SGC-7901 cells were measured using 'SRB assay'.[9] As shown in Table 1, β-elemene inhibited cell growth with an $IG_{50}$ of 260 and 230 μM in SGC-7901 and HeLa cells, respectively. Introduction of a piperazine (**IIa–IIi**), a piperidine (**IIj**), a morpholine (**IIk**), a tetrahydropyrrole (**IIl**), a thiophenylethylamine (**IIm**) or a cyclohexamine (**IIn**) has improved the anti-proliferative

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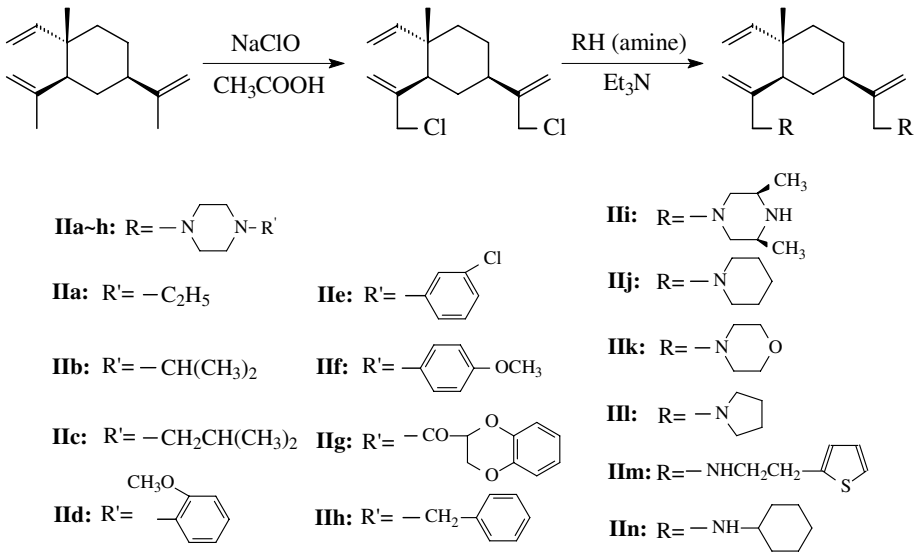


**Scheme 1.** The synthetic route and substitutes of β-elemene derivatives.

**Table 1.** The growth inhibitory effect of β-elemene derivatives on SGC-7901 and HeLa cells

| Compound | $IG_{50}$ (μM) | |
|---|---|---|
| | SGC-7901 | HeLa |
| β-Elemene | 259.5 ± 24.1 | 230.7 ± 18.5 |
| **IIa** | 26.9 ± 5.5 | 37.8 ± 7.17 |
| **IIb** | 18.1 ± 4.0 | 29.8 ± 4.3 |
| **IIc** | 102.1 ± 3.2 | 53.0 ± 0.57 |
| **IId** | 220.0 ± 2.1 | 142.8 ± 7.0 |
| **IIe** | >300 | >300 |
| **IIf** | 39.7 ± 0.68 | 70.2 ± 3.6 |
| **IIg** | 49.1 ± 1.2 | 57.6 ± 6.1 |
| **IIh** | 135.5 ± 1.3 | 54.3 ± 2.6 |
| **IIi** | 3.8 ± 0.64 | 3.2 ± 0.44 |
| **IIj** | 135.5 ± 16.2 | 42.8 ± 7.7 |
| **IIk** | 63.6 ± 0.96 | 60.0 ± 5.9 |
| **IIl** | 79.1 ± 11.5 | 54.5 ± 7.3 |
| **IIm** | 6.2 ± 1.1 | 5.9 ± 0.72 |
| **IIn** | 12.0 ± 5.1 | 8.8 ± 0.44 |

HeLa and SGC-7901 cells were treated with indicated compounds for 48 h and cell growth inhibition was measured with a SRB assay. The data shown are means plus SD, $n = 9$.

effect comparing with that of β-elemene. The $IG_{50}$s of these compounds in HeLa and SGC-7901 cells are listed in Table 1. Among these derivatives, compounds with a substitute of *cis*-2,6-dimethylpiperazine (**IIi**), 2-thiophenylethylamine (**IIm**), or cyclohexamine (**IIn**) were the most effective agents. Although compounds **IIa**–**IIh** (substituted with a piperazine) had an increased activity than that of β-elemene, further modification of piperazine structure with additional introduction of an ethyl (**IIa**), an isopropyl (**IIb**), a *p*-methoxyphenyl (**IIf**), or a benzodioxan-2-ylcarbonyl (**IIg**), group had a greater tumor growth inhibitory effect than the compounds with further introduction of an isobutyl (**IIc**), a *o*-methoxyphenyl (**IId**), a chlorophenyl (**IIe**), or a benzyl (**IIh**), group. Since compounds **IIi**, **IIm**, and **IIn** containing a secondary amino moiety are the most effective agents of inhibiting tumor cell growth, it suggests that the secondary amino group should contribute to their tumor growth inhibitory effect.

To further investigate the effect of compounds **IIi**, **IIm**, and **IIn**, cell growth inhibition and cytotoxicity were measured in leukemia K562 cells. As shown in Figure 1, compounds **IIi**, **IIm**, and **IIn** strongly inhibited growth of K562 cells. The $IG_{50}$s of compounds **IIi**, **IIm**, and **IIn** in K562 cells were 1.7, 1.3, and 3.7 μM, respectively. Trypan blue exclusion assay indicated that K562 cells did not lose viability after treatment with these compounds at such lower concentrations. To test whether these compounds inhibit cell growth through inducing apoptosis, apoptotic cells were determined by morphologic evaluation in cells stained with acridine orange (AO) and ethidium bromide (EB).[10] Neither of these compounds induces apoptosis in K562 cells at a concentration below 5 μM. These results suggest that the observed growth inhibitory effect of these substituted

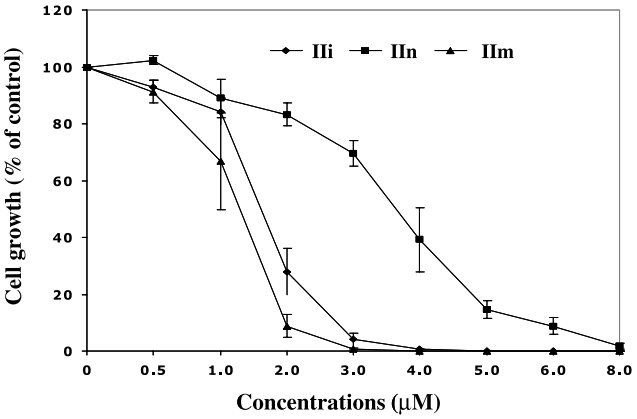


**Figure 1.** Compounds **IIi**, **IIm**, and **IIn** inhibit K562 cell growth. K562 cells were treated with the indicated compounds at labeled concentrations for 3 days. Cell number was counted and a growth inhibition was calculated by comparing with that of untreated cells.

β-elemenes may be through a cell death-independent pathway.

Recently, it has been shown that AKT-mTOR pathways play important roles in cell growth regulation.[11] mTOR proteins are serine/threonine kinases that control cell growth and proliferation in response to nutrient and growth factor stimulation.[12] AKT/mTOR pathway is frequently abnormal in a variety of tumors and mTOR inhibitors have shown promising effect of inhibiting tumor cell growth.[12] The effects of these β-elemene derivatives on AKT-mTOR pathways were measured in K562 cells. K562 cells were treated with compounds **IIi**, **IIm**, and **IIn** at the concentrations that inhibited cell growth and the phosphorylated AKT, mTOR, and p70S6K were measured using Western blot analysis. As shown in Figure 2A, compounds **IIm** and **IIn** at concentrations of 2 and 4 μM inhibited the phosphorylation of AKT, mTOR, and p70S6K. Since p-mTOR is a target of p-AKT and p-p70S6K is a target of p-mTOR,[13] these results suggest that the activities of both mTOR and AKT are inhibited by compounds **IIm** and **IIn**. Interestingly, although p-AKT and p-mTOR were not inhibited by compound **IIi** treatment, the p-p70S6K was evidently inhibited (Fig. 2A). This result suggests that compound **IIi** inhibits the activity of mTOR but not AKT. This observation is consistent with a recent report showing that inhibition of mTOR would not need AKT inhibition.[13] The time-dependent effect of compound **IIm** on the phosphorylation of mTOR and p70S6K was compared in K562 cells. As shown in Figure 2B, Compound **IIm** decreased the level of p-p70S6K as short as 2 h after treatment. The evident inhibition on the levels of p-mTOR was observed only at treatments after 16 h.

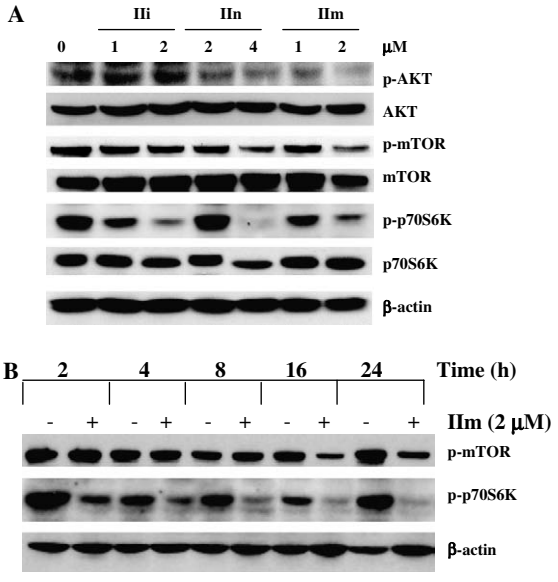


**Figure 2.** Compounds **IIi**, **IIm**, and **IIn** inhibit phosphorylation of AKT, mTOR, and p70S6K in K562 cells. (A) K562 cells were treated with **IIi** (1 and 2 μM), **IIm** (1 and 2 μM), and **IIn** (2 and 4 μM) for 24 h. (B) K562 cells were treated with **IIm** 2 μM for different times (2–24 h). Whole cellular protein lysates were prepared with RIPA buffer and Western blot analysis was used to analyze the protein levels using indicated antibodies. β-Actin was used as loading control.

Taken together, these data suggest that inhibition of mTOR activity might contribute to cell growth inhibitory effects of these compounds and that they might be novel mTOR inhibitors.

In summary, fourteen β-elemene derivatives have been synthesized. Among these derivatives, compounds **IIi**, **IIm**, and **IIn** are the most potent agents of inhibiting tumor cell growth. The inhibitory effect of compounds **IIi**, **IIm**, and **IIn** on tumor cell growth is not due to induction of cell death. It seems that these compounds inhibit cell growth through mTOR and/or AKT activity inhibition.

## 3. Materials and methods

### 3.1. Chemical synthesis

**3.1.1. Materials.** IR spectra were recorded on a Bruker IR-FIS-55 spectrophotometer model. IR spectra were obtained with potassium bromide pellets ($\nu_{max}$ in $cm^{-1}$). $^1H$ NMR spectra were recorded on Bruker ARX-300 MHz NMR instrument using tetramethylsilane (TMS) as the internal standard (chemical shift in $\delta$, ppm) in deuteriochloroform. Mass spectra were determined on an Agilent-1100 spectrometer and via a direct inlet probe in ESI mode. The purity of these compounds was evaluated by thin-layer chromatography (TLC) and by elemental analysis (C, H, and N). Plates for TLC were prepared with silica gel G and iodine vapor was used to develop the plates. Elemental analyses were carried out on a Perkin-Elmer-2400 elemental analyzer. The amine nucleophiles used in the experiments were obtained from commercial sources and used without further purification.

**3.1.2. Synthesis**

**3.1.2.1. 13,14-Dichloro-β-elemene (I).** Compound **I** was prepared according to the procedures described in the literature.[8] To a solution of β-elemene (26.0 g, 0.125 mol) in ice acetic acid (40 mL), 1.41 N sodium hypochlorite (198 mL, 0.275 mol) was added in over 4 h period under vigorous stirring at 5 °C. Then the mixture was stirred for 1 h at room temperature and extracted with petroleum ether (4× 50 mL). The combined organic extracts were washed with water (30 mL), dried over anhydrous sodium sulfate, and filtered. The filtrate was concentrated in vacuo to give the mixture as a brown oil, which was purified on a silica gel column with petroleum ether as eluent to give the title compound (11.2 g, 54.6% yield, $R_f = 0.2$, petroleum ether) as a yellow oil. $^1H$ NMR 0.97 (3H, s, $-CH_3$), 1.41–1.79 (6H, m, $-CH_2-$), 2.02 (2H, m, >CH–), 3.91 (2H, s, $-CH_2$-Cl), 3.99 (2H, s, $-CH_2$-Cl), 4.82–5.20 (6H, m, $=CH_2$), 5.80 (1H, dd, $-CH=CH_2$); IR (KBr): 3082, 2924, 2850, 1639, 1460, 1373, 903. MS (ESI) [M+1] (*m*/*z*) 273.4.

**3.1.3. General procedure for the preparation of compounds IIa–IIn.** A solution of **I** (2 mmol), amine (8 mmol), and triethylamine (8 mmol) in 10 mL dry *N*,*N*-dimethylformamide was refluxed for 8–20 h. Then water (10 mL) was added and the mixture was extracted with ethyl ether (4× 30 mL). The com-

bined organic extracts were dried over anhydrous sodium sulfate and filtered. The filtrate was concentrated in vacuo. The residue was purified on a silica gel column with petroleum ether–acetone as eluent to give a target product.

**3.1.3.1. 13,14-bis(4-Ethyl-1-piperazinyl)-β-elemene (IIa).** Compound was obtained in 46.2% yield. $^1$H NMR (DMSO-$d_6$) 0.96 (3H, s, –$CH_3$), 1.19 (6H, t, –$CH_3$), 1.41 (4H, m, –$CH_2$–), 1.61 (2H, m, –$CH_2$–), 2.02 (2H, m, >CH–), 2.50 (4H, m, –N-$CH_2$–), 2.73–3.13 (16H, m, –N-$CH_2$–), 3.25 (4H, m, –N-$CH_2$–), 4.85–5.10 (6H, m, =$CH_2$), 5.77 (1H, dd, –C*H*=$CH_2$); IR (KBr): 3080, 2925, 2854, 1638, 1457, 1375, 1320, 1147, 1075, 903. MS (ESI) [M+1] (*m*/*z*) 429.4. Calcd for $C_{27}H_{48}N_4$: C, 75.65; H, 11.29; N, 13.07. Found: C, 75.54; H, 11.31; N, 13.11.

**3.1.3.2. 13,14-bis(4-Isopropyl-1-piperazinyl)-β-elemene (IIb).** Compound was obtained in 43.2% yield. $^1$H NMR 1.04 (3H, s, –$CH_3$), 1.10 (12H, d, –CH-C*H*$_3$), 1.36–1.79 (6H, m, –$CH_2$–), 2.02–2.78 (20H, m, >CH–, –N-$CH_2$–), 3.11 (4H, m, –N-$CH_2$–), 4.72–5.15 (6H, m, =$CH_2$), 5.85 (1H, dd, –C*H*=$CH_2$); IR 3080, 2965, 2807, 1639, 1452, 1335, 1179, 1014, 903. MS (ESI) [M+1] (*m*/*z*) 457.4. Calcd for $C_{29}H_{52}N_4$: C, 76.26; H, 11.47; N, 12.27. Found: C, 76.48; H, 11.46; N, 12.19.

**3.1.3.3. 13,14-bis(4-Isobutyl-1-piperazinyl)-β-elemene (IIc).** Compound was obtained in 40.5% yield. $^1$H NMR(DMSO-$d_6$) 0.93 (12H, d, –CH-C*H*$_3$), 0.95 (3H, s, –$CH_3$), 1.23–1.42 (4H, m, –$CH_2$–), 1.63 (2H, m, –$CH_2$–), 1.99 (4H, m, >CH–), 2.50–3.66 (24H, m, –N-$CH_2$–), 4.84–5.14 (6H, m, =$CH_2$), 5.81 (1H, dd, –C*H*=$CH_2$); IR 3080, 2951, 2805, 1639, 1458, 1377, 1295, 1013, 903, 831. MS (ESI) [M+1] (*m*/*z*) 485.4. Calcd for $C_{31}H_{56}N_4$: C, 76.80; H, 11.64; N, 11.56. Found: C, 76.67; H, 11.67; N, 11.72.

**3.1.3.4. 13,14-bis[4-(2-Methoxyphenyl)-1-piperazinyl]-β-elemene (IId).** Compound was obtained in 39.7% yield. $^1$H NMR 1.07 (3H, s, –$CH_3$), 1.47–1.72 (6H, m, –$CH_2$–), 2.28 (3H, m, >CH–, –N-$CH_2$–), 2.65–2.82 (9H, m, –N-$CH_2$–), 3.03–3.18 (10H, m, –N-$CH_2$–), 3.89–3.90 (6H, s, –O-$CH_3$), 4.87–5.14 (6H, m, =$CH_2$), 5.88 (1H, dd, –C*H*=$CH_2$), 6.88–7.05 (8H, m, Ar-H); IR 3077, 2934, 2812, 1639, 1594, 1500, 1241, 1012, 904, 746. MS (ESI) [M+1] (*m*/*z*) 585.4. Calcd for $C_{37}H_{52}N_4O_2$: C, 75.99; H, 8.96; N, 9.58. Found: C, 76.12; H, 8.98; N, 9.55.

**3.1.3.5. 13,14-bis[4-(3-Chlorophenyl)-1-piperazinyl]-β-elemene (IIe).** Compound was obtained in 37.1% yield. $^1$H NMR 1.05 (3H, s, –$CH_3$), 1.50–1.69 (8H, m, –$CH_2$–, >CH–), 2.23 (1H, d, –N-$CH_2$–), 2.24–2.59 (8H, m, –N-$CH_2$–), 2.73 (1H, d, –N-$CH_2$–), 3.00 (2H, s, –N-$CH_2$–), 3.15 (8H, br, –N-$CH_2$–), 4.85–5.11 (6H, m, =$CH_2$), 5.80 (1H, dd, –C*H*=$CH_2$), 6.74–6.88 (6H, m, Ar-H), 7.16 (2H, m, Ar-H); IR 3079, 2930, 2818, 1638, 1594, 1594, 1565, 1236, 1009, 904, 765. MS (ESI) [M+1] (*m*/*z*) 593.7. Calcd for $C_{35}H_{46}N_4Cl_2$: C, 70.81; H, 7.81; N, 9.44. Found: C, 70.79; H, 7.83; N, 9.40.

**3.1.3.6. 13,14-bis[4-(4-Methoxyphenyl)-1-piperazinyl]-β-elemene (IIf).** Compound was obtained in 42.8% yield. $^1$H NMR 1.02 (3H, s, –$CH_3$), 1.51–1.78 (6H, m, –$CH_2$–), 2.18 (2H, m, >CH–), 2.26–2.73 (10H, m, –N-$CH_2$–), 2.99–3.07 (10H, m, –N-$CH_2$–), 3.76 (6H, s, –O-$CH_3$), 4.28–5.08 (6H, m, =$CH_2$), 5.80 (1H, dd, –C*H*=$CH_2$), 6.81–6.85 (8H, m, Ar-H); IR 3080, 2941, 2814, 1638, 1511, 1451, 1249, 1037, 1012, 901, 821. MS (ESI) [M+1] (*m*/*z*) 585.4. Calcd for $C_{37}H_{52}N_4O_2$: C, 75.99; H, 8.96; N, 9.58. Found: C, 75.87; H, 8.92; N, 9.60.

**3.1.3.7. 13,14-bis[4-(1,4-Benzodioxan-2-ylcarbonyl)-1-piperazinyl]-β-elemene (IIg).** Compound was obtained in 27.5% yield. $^1$H NMR 1.02 (3H, s, –$CH_3$), 1.49–2.09 (8H, m, –$CH_2$–, >CH–), 2.48 (8H, br, –N-$CH_2$–), 2.88–3.11 (4H, m, –N-$CH_2$–), 3.60 (4H, br, –N-$CH_2$–), 3.73 (4H, br, –N-$CH_2$–), 4.28–4.50 (5H, m, –O-$CH_2$–, –O-CH<), 4.81–5.30 (7H, m, =$CH_2$, –O-CH–), 5.78 (1H, dd, –C*H*=$CH_2$), 6.89 (8H, d, Ar-H,); IR 3080, 2929, 2808, 1713, 1645, 1595, 1493, 1268, 1225, 1003, 909, 750. MS (ESI) [M+1] (*m*/*z*) 697.4. Calcd for $C_{41}H_{52}N_4O_6$: C, 70.66; H, 7.52; N, 8.04. Found: C, 70.83; H, 7.55; N, 7.98.

**3.1.3.8. 13,14-bis(4-Benzyl-1-piperazinyl)-β-elemene (IIh).** Compound was obtained in 46.4% yield. mp 47.1–50.8 °C. $^1$H NMR 0.98 (3H, s, –$CH_3$), 1.39–1.60 (6H, m, –$CH_2$–), 1.72 (2H, s, >CH–), 2.45 (16H, br, –N-$CH_2$–), 2.65 (1H, d, –N-$CH_2$–), 2.90 (2H, s, –N-$CH_2$–), 3.02 (1H, d, –N-$CH_2$–), 3.51 (4H, s, –C$H_2$-Ph), 4.76–5.03 (6H, m, =$CH_2$), 5.81 (1H, dd, –C*H*=$CH_2$), 7.24–7.31 (10H, m, Ar-H); IR 3080, 2938, 2804, 1640, 1456, 1293, 1134, 1010, 897, 742. MS (ESI) [M+1] (*m*/*z*) 553.4. Calcd for $C_{37}H_{52}N_4$: C, 80.39; H, 9.48; N, 10.13. Found: C, 80.12; H, 9.51; N, 10.09.

**3.1.3.9. 13,14-bis(*cis*-3,5-Dimethyl-1-piperazinyl)-β-elemene (IIi).** Compound was obtained in 63.8% yield.$^1$H NMR 1.00 (15H, m, –$CH_3$), 1.40–1.65 (8H, m, –$CH_2$–, >CH–), 2.09 (4H, m, –N-$CH_2$–), 2.60–2.98 (12H, m, –N-$CH_2$–, –N-CH<), 4.78–5.03 (6H, m, =$H_2$), 5.80 (1H, dd, –C*H*=$CH_2$); IR 3080, 2925, 2854, 1638, 1457, 1375, 1147, 1075, 903. MS (ESI) [M+1] (*m*/*z*) 429. Calcd for $C_{27}H_{48}N_4$: C, 75.65; H, 11.29; N, 13.07. Found: C, 75.78; H, 11.27; N, 13.04.

**3.1.3.10. 13,14-bis(1-Piperdinyl)-β-elemene (IIj).** Compound was obtained in 78.2% yield. $^1$H NMR 0.97 (3H, s, –$CH_3$), 1.52–1.69 (7H, m, –$CH_2$–), 1.83–1.91 (6H, m), 2.03–2.37 (6H, m, –$CH_2$–, >CH–), 2.63–3.03 (6H, m, –$CH_2$–, –N-$CH_2$–), 3.47–3.60 (6H, m, –N-$CH_2$–), 4.02 (1H, m, –N-$CH_2$–), 4.90–5.62 (6H, m, =$CH_2$), 5.75 (1H, dd, –C*H*=$CH_2$); IR 3078, 2938, 2857, 1639, 1583, 1449, 1057, 1009, 930, 890. MS (ESI) [M+1] (*m*/*z*) 371. Calcd for $C_{25}H_{42}N_2$: C, 81.02; H, 11.42; N, 7.56. Found: C, 81.30; H, 11.35; N, 7.56.

**3.1.3.11. 13,14-bis(*N*-Morpholinyl)-β-elemene (IIk).** Compound was obtained in 68.7% yield. $^1$H NMR 1.01 (3H, s, –$CH_3$), 1.41–1.64 (6H, m, –$CH_2$–), 2.01–2.38 (10H, m, >CH–, –N-$CH_2$–), 2.68 (1H, d, –N-$CH_2$–), 2.91 (2H, s, –N-$CH_2$–), 3.02–3.06 (1H, d, –N-$CH_2$–), 3.69 (8H, s, –O-$CH_2$–), 4.80–5.06 (6H, m,

=$CH_2$), 5.77–5.86 (1H, dd, –C*H*=$CH_2$); IR 3080, 2927, 2852, 1639, 1453, 1292, 1118,1010, 906, 865. (MS (ESI) [M+1] (*m*/*z*) 375. Calcd for $C_{23}H_{38}N_2O_2$: C, 73.75; H, 10.23; N, 7.48. Found: C, 73.78; H, 10.17; N, 7.40.

**3.1.3.12. 13,14-bis(1-Tetrahydropyrrolyl)-β-elemene (III).** Compound was obtained in 75.1% yield. $^1$H NMR 1.00 (3H, s, –$CH_3$), 1.40–1.60 (6H, m, –$CH_2$–), 1.76 (7H, br, –$CH_2$–), 2.06 (3H, m, –$CH_2$–, >CH–), 2.30 (1H, m, –N-$CH_2$–), 2.46 (7H, br, –N-$CH_2$–), 2.70 (1H, d, –N-$CH_2$–), 3.03 (1H, d, –N-$CH_2$–), 3.06 (1H, d, –N-$CH_2$–), 3.23 (1H, d, –N-$CH_2$–), 4.70–5.07 (6H, m, =$CH_2$), 5.77–5.86 (1H, dd, –C*H*=$CH_2$); IR 3080, 2926, 2782, 1639, 1460, 1373, 1126, 1007, 901. MS (ESI) [M+1] (*m*/*z*) 343. Calcd for $C_{23}H_{38}N_2$: C, 80.64; H, 11.48; N, 8.18. Found: C, 79.78; H, 11.27; N, 8.14.

**3.1.3.13. 13,14-bis[2-(2-Thiophenyl)ethylamino]-β-elemene (IIm).** Compound was obtained in 46.2% yield. $^1$H NMR 0.98 (3H, s, –$CH_3$), 1.35 (1H, m, –$CH_2$–), 1.54–1.68 (4H, m, –$CH_2$–), 2.09 (1H, br, –$CH_2$–), 2.30 (1H, br, >CH–), 2.55 (1H, br, –CH<), 3.18–4.01 (12H, m, –$CH_2$-N-$CH_2$-$CH_2$–), 4.89–5.58 (6H, m, =$CH_2$), 5.75 (1H, dd, –C*H*=$CH_2$), 6.93 (4H, d, –CH=), 7.16 (2H, d, –S-CH=); IR 3420, 2928, 1641, 1438, 1013, 918, 850. MS (ESI) [M+1] (*m*/*z*) 455. Calcd for $C_{27}H_{38}N_2S_2$: C, 73.75; H, 8.48; N, 3.19. Found: C, 73.86; H, 8.37; N, 3.25.

**3.1.3.14. 13,14-bis(Cyclohexamino)-β-elemene (IIn).** Compound was obtained in 76.9% yield. $^1$H NMR 1.01 (3H, s, –$CH_3$), 1.24–1.35 (7H, m, –$CH_2$–), 1.56–1.69 (13H, m, –$CH_2$–), 2.14–2.35 (7H, m, –$CH_2$–, >CH–), 2.54 (1H, d, >CH–), 2.96 (2H, m, –N-$CH_2$–), 3.40 (1H, m, –N-$CH_2$–), 3.52 (1H, m, –N-$CH_2$–), 3.69 (1H, m, –N-CH<), 3.88 (1H, m, –N-CH<), 4.96–5.86 (6H, m, =$CH_2$), 5.78 (1H, dd, –C*H*=$CH_2$); IR 3345, 3084, 2940, 2863, 1638, 1511, 1441, 1249, 1147, 1039, 915, 821. MS (ESI) [M+1] (*m*/*z*) 399. Calcd for $C_{27}H_{46}N_2$: C, 81.34; H, 11.63; N, 7.03. Found: C, 81.58; H, 11.71; N, 7.06.

### 3.2. Biological activity

**3.2.1. SRB assay.** SRB assay was performed according to reported method.[9] Briefly, HeLa and SGC-7901 cells ($2 \times 10^3$) were plated in each well of a 96-well plate and were allowed to adhere and spread for 24 h. Then various concentrations of each compound were added and cultured for another 2 days. Cells in the plates were fixed with addition of 50 μL of 50% cold trichloroacetic acid for 1 h at 4 °C. The plates were then drained, rinsed five times with water, and air-dried. Fifty microliters of 0.4% sulforhodamine B (SRB) solution was added in each well and the plates were incubated for an additional 0.5 h. Unbound dye was drained and removed by washing four times with 1% glacial acetic acid. The dye in cells was dissolved by adding 150 μL/well of 10 mM Tris-base, pH 10.5, and an absorbance at 540 nm was measured in a 96-well plate reader. Growth inhibition was determined as compared to that of untreated cells (%) and an $IG_{50}$ (concentration of inhibiting 50% cell growth) was calculated.

**3.2.2. Cell viability and apoptosis assay.** K562 cells were cultured in RPMI-1640 medium supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin, and 10% heat-inactivated fetal bovine serum. Cells in logarithmic growth were seeded at $1 \times 10^5$ cells/mL and were treated with different compounds **IIi**, **IIm**, and **IIn** at different concentrations for 3 days. Studies were performed in triplicate. The growth inhibition in cells after treatment with different concentrations was calculated comparing with control cells and a half growth inhibitory concentration ($IG_{50}$) was obtained by regression analysis of the concentration–response data. Cell viability was determined after staining with Trypan blue. Trypan blue-stained (non-viable) cells and total cell number were determined with the aid of a hematocytometer. Apoptotic cells were determined by morphologic evaluation in cells stained with AO and EB, and assessed by fluorescence microscopy as described previously.[10] Briefly, 1 μL of stock solution containing 100 μg/mL AO and 100 μg/mL EB was added to 25 μL of cell suspension. Apoptotic cells (identified by nuclear shrinkage, blebbing, and the presence of apoptotic bodies) in total 300 cells were counted using fluorescence microscopy, and the percentage of apoptotic cells was calculated.

**3.2.3. Western blot analysis.** Western blot analysis was done as described before.[10] Generally, protein extracts (50 μg) of **IIi**, **IIm** or **IIn** treated K562 cells were prepared with RIPA lysis buffer [50 mmol/L Tris–HCl, 150 mmol/L NaCl, 0.1% SDS, 1% NP40, 0.5% sodium deoxycholate, 1 mmol/L phenylmethylsulfonyl fluoride, 100 μmol/L leupeptin, and 2 μg/mL aprotinin (pH 8.0)] and were subjected to electrophoresis on 8–12% SDS–polyacrylamide gels. The separated proteins were then transferred to nitrocellulose membranes that were stained with 0.2% Ponceau S red to assure equal protein loading and transfer. After blocking with 5% non-fat milk, the membranes were incubated with a monoclonal antibody to AKT, mTOR, p70S6K, phospho-AKT (p-AKT, $Ser^{473}$), phospho-mTOR (p-mTOR, $Ser^{2448}$) or phospho-p70S6K (p-p70S6K, $Thr^{389}$) (the antibodies were purchased from Cell Signalling Technology Inc., Beverly, MA). Immunocomplexes were visualized by chemiluminescence (ECL kit, Amersham Biosciences Corp., Piscataway, NJ).

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