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Synthesis of oleanolic acid hydrazide-hydrazone hybrid derivatives and investigation of their cytotoxic effects on A549 human lung cancer cells

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ABSTRACT

In this study, 13 new hybrid compounds were synthesized starting from the natural product oleanolic acid and their *in vitro* cytotoxic activities were investigated on the BEAS-2B and A549 cell lines. For this purpose, initially, the secondary OH group at the C3 position of oleanolic acid was protected as methoxy and the carboxyl group was converted to a hydrazide. The hybrid compounds **12(a-m)** were synthesized starting from the above hydrazide and using 13 different aromatic aldehydes. The biological activity of the resulting compounds was evaluated with doxorubicin as the standard. The IC_{50} values of the cytotoxic effects of doxorubicin on the BEAS-2B and A549 cells are 0.09 and 0.14 μM, respectively. The hybrid compounds with the lowest cytotoxicity on the BEAS-2B cells were **12b** (IC_{50} = 2.96 μM) and **12f** (IC_{50} = 2.53 μM). The corresponding cytotoxic activity of the same two compounds on A549 cells was 0.08 and 0.22 μM, respectively. Compound **12b** was found to have an equivalent cytotoxic activity to doxorubicin on the A549 cells, whereas its activity on the BEAS-2B cells was determined to be approx. 32 times as less potent as doxorubicin. According to the *in vitro* test results, the hybrid compound **12b** may be a promising candidate for further investigation as an anticancer agent.

Introduction

Cancer is a disease that occurs with uncontrolled cell division in an organ or tissue. It has been reported that the term cancer was first used by Hippocrates in the 4th century BCE to describe new formations that could not be treated in the organism [1–3]. Cancer is an important health problem worldwide and one of the leading causes of death. Cancer caused about 10 million deaths in 2018. Globally, about one in six deaths are caused by cancer. According to the estimates of the World Health Organization in 2015, cancer was the first or second cause of death before the age of 70 in 91 of the 172 countries studied, and furthermore, the third or fourth cause of death in 22 other countries [4–7].

The global ranks and rates of the most prevalent 10 cancer types in either sex and in general in 2018 are given in Table 1 [8,9]. Lung cancer was the most common cancer in men worldwide, contributing 15.5% of the total number of new cases diagnosed in 2018, and breast cancer was the most common cancer in women worldwide, contributing 25.4% of the total number of new cases diagnosed in 2018.

Successful cancer treatment is very important for the increasing average life span of humans and the life quality of the patients [10–12].

Terpenes are secondary metabolites that constitute very important defense mechanisms of plants. Triterpenes have 30 carbons and generally have a cyclic structure [13]. Many biological activities are known of α-amyrin, uvaol, ursolic acid, β-amyrin, erythrodiol, oleanolic acid as well as betulinic acid and lupeol (Fig. 1) [14–18].

Abbreviations: BCE, Before Common Era; HCV, Hepatitis C Virus; HIV, Human Immunodeficiency Virus; NMR, Nuclear Magnetic Resonance; μM, Micromolar; HRMS, High Resolution Mass Spectrometry; ESI, Electrospray Ionization; HPLC, High Performance Liquid Chromatography; PDA, Photo Diode Array Detector; mL, Milliliter; DMEM, Dulbecco's Modified Eagle Medium; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide; TD_{50}, Half Maximum Toxic Dose; ED_{50}, Half Maximum Effective Dose; IC_{50}, Half Maximum Inhibitory Concentration; SD, Standard Deviation; TLC, Thin Layer Chromatography; FBS, Fetal Bovine Serum; BEAS-2B, Human Bronchial Epithelial Cells; A549, Adenocarcinomic Human Alveolar Basal Epithelial Cells; EDTA, Ethylene Diamine Tetra Acetic Acid; Rt, Retention Time.

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Protective effects of ursolic and oleanolic acids on the lung, kidney, liver, and brain have been reported. Oleanolic acid also exhibits antimicrobial properties on a large number of bacteria, human immunodeficiency virus (HIV), hepatitis C virus (HCV), and *Plasmodium* protozoa strains that cause malaria [19]. It has been determined in many studies that oleanolic acid and its synthetic derivatives have anti-cancer, antidiabetic, anti-arrhythmic, anti-hyperlipidemic, anti-microbial, anti-hypercholesterolemic, and anti-cardiovascular effects [20–32].

Drug discovery is quite difficult due to the amount of time and expense necessary for the discovery of new drugs. The average time of the development of a drug was 8 years in the 1960 s, but today it is 12–15 years [33,34]. Hybrid molecules can be defined as chemical compounds having different biological functions and two or more structural domains [35]. Different pharmacophores of a hybrid molecule can have activity on disparate biological targets. The aim of this approach in drug design is to determine and improve the biological activity of a molecule fragment by adding groups that can bind to the active sites of different enzymes or receptors [36–40].

Natural products and their semi-synthetic derivatives constitute a large percentage of drugs used for treatment of various diseases [41]. Due to the toxicity and poor bioavailability of anticancer drugs, it is sometimes required to improve the pharmacokinetic properties of new anticancer drugs in the treatment. Although an increasing number of anticancer agents have been developed day by day, their low selectivity and multi-drug resistance often prohibit a successful cancer treatment [42]. Therefore, medical professionals need new, potent, and selective anticancer agents [43–46]. The secondary metabolites of plants are traditionally used in the treatment of cancer. Nowadays semi-synthetic derivatives of natural products comprise the majority of anticancer agents [20,21,47–55,57,58].

Result and discussion

Chemistry

It has been reported in previous studies that both hydrazide and hydrazone compounds show a wide variety of biological activities [59–64]. When these structures are combined with natural products, potentially biologically active hybrid molecules are formed that can have dual or more effects [66–72]. For this purpose, in this study, 13 new hybrid compounds were synthesized from the natural product oleanolic acid and the *in vitro* cytotoxic activities of these compounds were investigated on the BEAS-2B human non-tumorigenic lung epithelial cell and the A549 adenocarcinomic human alveolar basal epithelial cell lines.

Initially, 3-methyl oleanolic acid was prepared by converting the OH group at the C3 position of the oleanolic acid to a methoxy group. With this reaction, possible acetal formations that would occur in reactions with aldehydes in later stages were prevented. Then, the hydrazide compound **(11b)** was synthesized from the acyl halide of oleanolic acid **(10).** Finally, hybrid compounds **12(a-m)** were synthesized starting from the hydrazide using 13 different aromatic aldehydes. During the synthesis of **11a** from acyl halide of 3-methyloleanolic acid **(10)** with hydrazine hydrate in dichloromethane at room temperature, the dimeric structure **11b** was obtained as a side-product **(**Scheme 1**).** In the ^{13}C NMR spectrum, while the chemical shift of the hydrazide carbonyl of **11a** is 179 ppm, this value is 172 ppm for compound **11b.**

Biological activity studies

The cytotoxic effects of the hybrid compounds were investigated against human non-tumorigenic lung epithelial cells (BEAS-2B) and adenocarcinomic human alveolar basal epithelial cells (A549). The relative cell viability of the BEAS-2B and A549 cells against the cytotoxic effects of five different concentrations [73,74] (0.1, 0.5, 1, 5, and 10 μM) of hybrid compounds were given in Figs. 2 and 3. The IC_{50} values of the cytotoxic effects of the hybrid compounds on the BEAS-2B and A549 cells are given in Table 2. Additionally, the inhibition graphics with the ± SD value of the cytotoxicity assays of the hybrid compounds are given in Supporting Information.

Conclusion

It has been reported in previous studies that both hydrazide and hydrazone compounds show a wide variety of biological activities [62,63,75]. In this study, hybridized triterpene/hydrazide-hydrazone structures were synthesized from oleanolic acid which is a biologically active natural product. In the synthetic studies, 13 new hybrid compounds shown in Scheme 1 were synthesized from oleanolic acid and the *in vitro* cytotoxic activities of these compounds were investigated on the BEAS-2B and A549 cell lines.

Biological activity results were compared to the standard doxorubicin. The IC_{50} values of the cytotoxic effect of doxorubicin on relative cell viability on the BEAS-2B and A549 cells are 0.09 and 0.14 μM respectively. The hybrid compounds with the lowest cytotoxicity on the BEAS-2B cells are **12e** (IC_{50} = 5.26 μM), **12b** (IC_{50} = 2.96 μM), **12c** (IC_{50} = 2.79 μM), **12f** (IC_{50} = 2.54 μM), **12d** (IC_{50} = 2.31 μM), **12 k** (IC_{50} = 1.55 μM), **12i** (IC_{50} = 1.52 μM) and **12j** (IC_{50} = 0.30 μM). When compared with doxorubicin, **12e**, **12b** and **12c** were 57, 33 and 31 times as less active in the same healthy cells as doxorubicin, respectively.

The IC_{50} values of the compounds **12e, 12b, 12c, 12f and 12d** were 1.72, 0.08, 0.35, 0.22 and 0.31 μM, respectively, against A549 cells. Although **12b** and doxorubicin showed the same toxicity on A549 cells, the toxicity of **12b** on healthy cells was approximately 32 times lower than doxorubicin. So the selectivity of certain hybrid compounds was higher than doxorubicin. The selectivity (TD_{50}/ED_{50}) of compounds **12b, 12f**, **12c** and doxorubicin were 37, 11.5, 7.9, 0.6, respectively. As a result, the most selective compounds were determined as **12b, 12f,** and **12c**.

In a study investigating the cytotoxic effects of oleanolic acid derivatives, the cytotoxic effects of the compounds against A549 cells were found in the range of 0.22–727 μM. Comparing the related study with

Table 1
The global cancer rates of the first 10 cancer types in both sexes in 2018.

	Both Sexes		Men		Women	
Rank	Cancer types	% all of the cancers	Cancer types	% all of the cancers	Cancer types	% all of the cancers
1	Lung	12.3	Lung	15.5	Breast	25.4
2	Breast	12.3	Prostate	14.5	Colorectal	9.7
3	Colorectal	10.6	Colorectal	11.4	Lung	8.8
4	Prostate	7.5	Stomach	7.8	Cervical	6.9
5	Stomach	6.1	Liver	6.8	Thyroid	6.3
6	Liver	5.0	Bladder	4.8	Endometrial	5.3
7	Esophagus	3.4	Esophagus	4.5	Stomach	4.3
8	Cervical	3.3	Lymphoma	3.2	Ovarian	3.6
9	Thyroid	3.3	Kidney	2.9	Liver	3.0
10	Bladder	3.2	Leukemia	2.8	Lymphoma	2.7

our study, it was determined that the compounds in our study were more selective against the A549 cells [76].

Today, due to the low bioavailability of numerous chemotherapeutics and their side effects such as toxicity, patients need new anticancer drugs in treatment. While an increasing number of anticancer agents are being developed everyday, some obstacles such as low selectivity and multi-drug resistance are significant barriers to successful cancer treatment. Therefore, there is a significant need for the discovery of novel, potent, and selective anticancer agents to kill tumor cells or decrease their proliferation. In this study, it was determined that the hybrid molecule **12b** compared with doxorubicin, which is used as an anticancer agent, maybe a more selective potential anticancer agent according to the *in vitro* test results. Because **12b** is more selective than doxorubicin toward the BEAS-2B and A549 cells.

Experimental section

Chemistry

All solvents, chemicals, and other supplies used in the experiments were purchased from Merck, Sigma Aldrich, TCI Chemicals, and other suppliers. Although commercially available chemicals and solvents had high purity, purification procedures were performed as described in the literature, when necessary [77–79].

In general, column chromatography was used in the chromatographic separations, and in some cases, the substance was crystallized using suitable solvents. Silica gel was used as the stationary phase and a mixture of ethyl acetate and hexane in certain proportions was used as the mobile phase in glass columns of different lengths and thicknesses. The experiments and column chromatography were monitored by thin-layer chromatography (TLC), and detection of spots was conducted using UV light, cerium(IV)sulfate solution 10% in sulfuric acid, and heating in the oven at 100 °C. Nuclear magnetic resonance (NMR) analyses (^{1}H NMR and ^{13}C APT NMR) were used for the determination of chemical structures. HRMS analyses were performed for the determination of molecular weight.

Melting points were determined by the Stuart SMP30 melting point apparatus. ^{1}H NMR and ^{13}C APT NMR spectra were recorded by Bruker Avance NEO NMR Spectrometer at 500 and 125 MHz, respectively. Coupling constant values were given in Hertz (Hz). Chemical shifts were reported in δ (parts per million) units relative to the internal standard tetramethylsilane ($\delta = 0.00$ ppm) and the peak splits were described as follows: s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), bs (broad singlet), dd (doublet of doublets) and dt (doublet of triplets). HRMS spectra were recorded using the ESI technique by Thermo Fischer Scientific Q Exactive™ Hybrid Quadrupole-Orbitrap™ Mass Spectrometer. HPLC chromatograms were recorded using the Waters preparative HPLC and PDA detector.

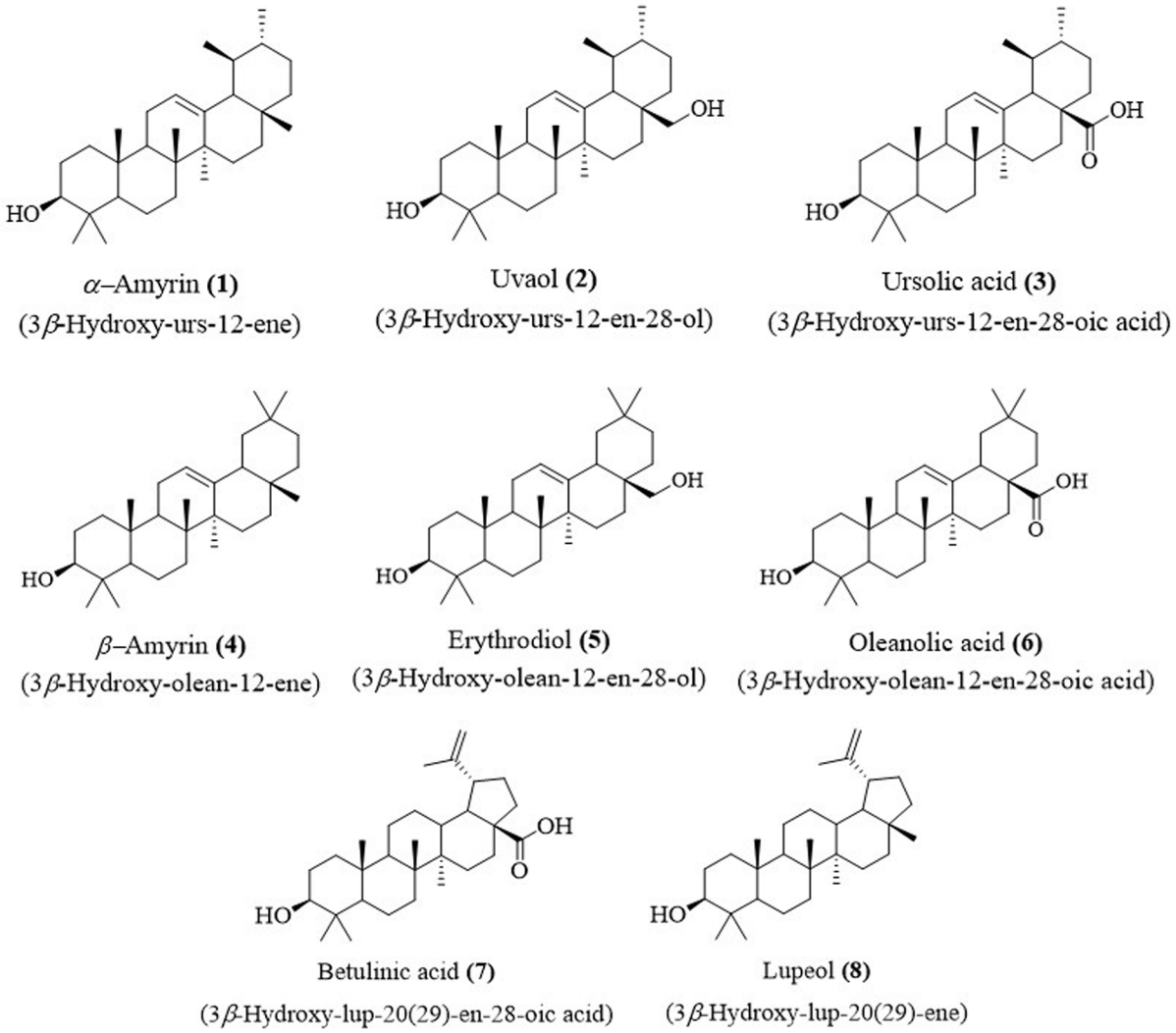
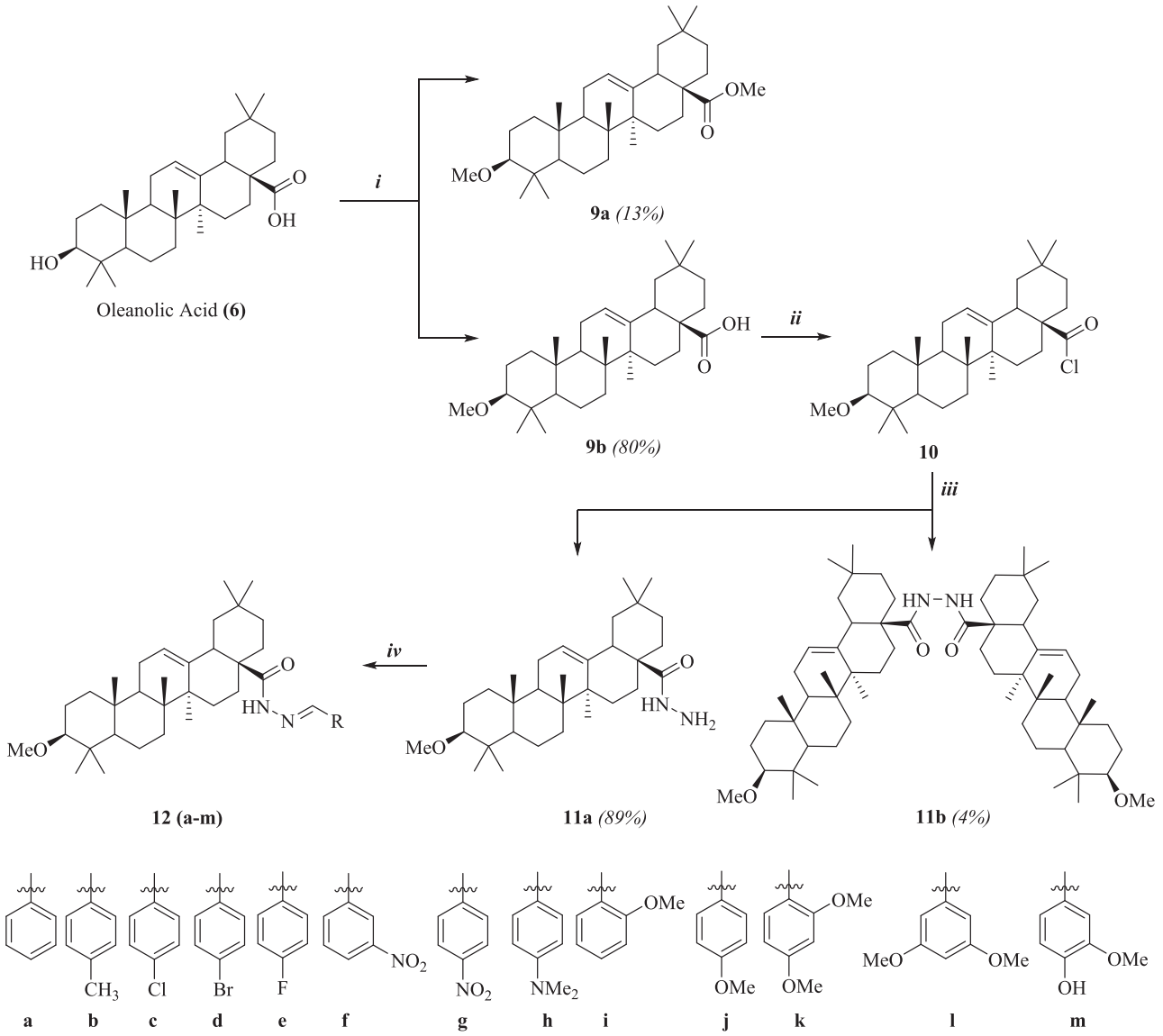


Fig. 1. Some bioactive oleanane, ursane, and lupane triterpenoids.



Reagent and Conditions: ***i)*** NaH, MeI, THF, 25°C, N_2, 12h; ***ii)*** Oxalyl Chloride, DCM, 25°C, N_2, 24h, 98%; ***iii)*** $NH_2NH_2{\cdot}H_2O$, DCM, 25°C, N_2, 5h; ***iv)*** R-CHO, HOAc, MeCN/$CHCl_3$, 65°C, 24h

Scheme 1.

Synthesis of methyl derivatives of oleanolic acid (9a and 9b)

A round-bottomed flask was charged with freshly distilled tetrahydrofuran (THF) and sodium hydride (NaH) (5.25 g, 130 mmol, 3 equiv.). Oleanolic acid (20 g, 44 mmol, 1 equiv.) was added and stirred for 30 min at room temperature. Methyl iodide (MeI) (3.5 mL, 55 mmol, 1.25 equiv.) was added and the resulting mixture was stirred overnight under an inert atmosphere. At completion, the excess sodium hydride was destroyed with water (15 mL). The solvent was removed under reduced pressure. The residue was washed with water (3x300 mL) and extracted with chloroform (3x300 mL). The organic layers were combined, dried over Na_2SO_4, and filtered. The solvent was removed under reduced pressure and the residue was adsorbed on silica gel. The products were purified by silica gel column chromatography using an ethyl acetate and hexane mixture (1:9). The dimethyl derivative of oleanolic acid **(9a)** eluted first (white solid, 2.75 g, 13% yield) and the 3-methyl-oleanolic acid **(9b)** (white solid, 17 g, 80% yield) eluted more slowly.

*Compound **9a:*** m.p.: 156 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ: 5.28 (t, J = 3.40 Hz, 1H), 3.61 (s, 3H), 3.34 (s, 3H), 2.85 (dd, J = 4.40, 13.80 Hz, 1H), 2.65 (dd, J = 4.00, 11.60 Hz, 1H), 1.00 (s, 3H), 0.89 (s, 3H), 0.88 (s, 3H), 0.86 (s, 3H), 0.79 (d, J = 6.50 Hz, 3H), 0.69 (s, 3H), 0.68 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ: 177.60, 143.10, 121.70, 87.90, 56.80, 55.00, 50.80, 46.90, 46.00, 45.20, 40.90, 40.60, 38.60, 38.00, 37.60, 36.30, 33.10, 32.40, 31.90, 31.70, 30.00, 27.40, 27.00, 25.20, 22.90, 22.70, 22.30, 21.30, 17.50, 16.10, 15.60, 14.60. HRMS (ESI) calculated m/z for $C_{32}H_{52}O_3$ $[M + H]^+$ 485.39947, found 485.39954.

*Compound **9b:*** m.p.: 210 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.20 (t, J = 3.70 Hz, 1H), 3.28 (s, 3H), 2.77 – 2.72 (m, 1H), 2.59 (dd, J = 11.70, 4.30 Hz, 1H), 1.05 (s, 3H), 0.89 (s, 3H), 0.86 (s, 3H), 0.84 (s, 4H), 0.83 (s, 3H), 0.67 (d, J = 6.40 Hz, 6H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 184.53, 143.63, 122.67, 88.74, 57.57, 55.80, 47.65, 46.55, 45.89, 41.54, 40.88, 39.31, 38.71, 38.32, 37.12, 33.80, 33.09, 32.60, 32.44, 30.68, 28.12, 27.68, 25.96, 23.60, 23.44, 22.88, 22.02, 18.17, 17.19, 16.29, 15.29. HRMS (ESI) calculated m/z for $C_{31}H_{50}O_3$ $[M + H]^+$ 471.38382, found 471.38281; HPLC-PDA: λ max, MeCN:MeOH (1:1), Rt: 17.77 min, 94.7%.

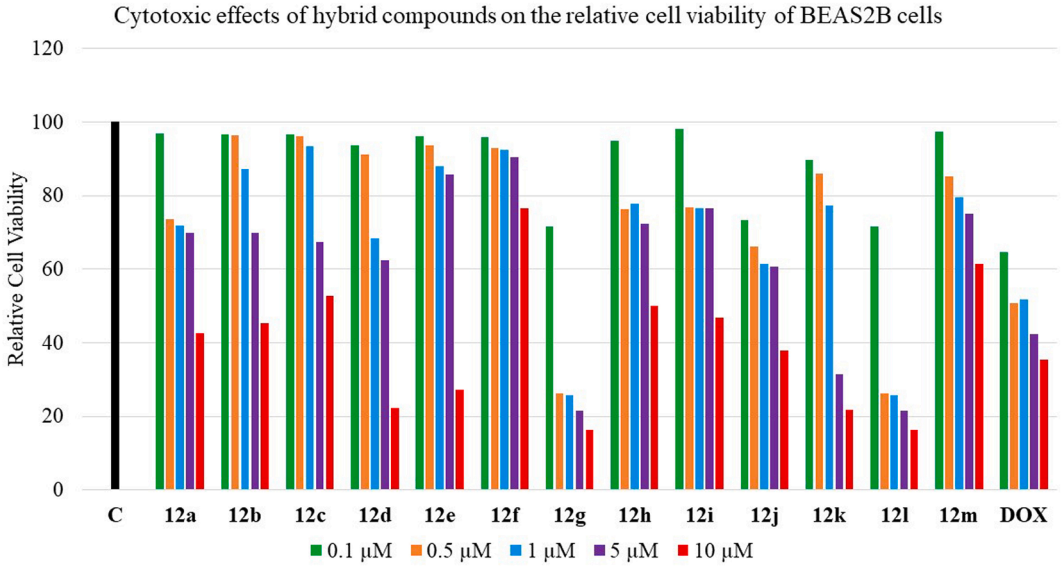


Fig. 2. Cytotoxic effects of the hybrid compounds on BEAS-2B cells.

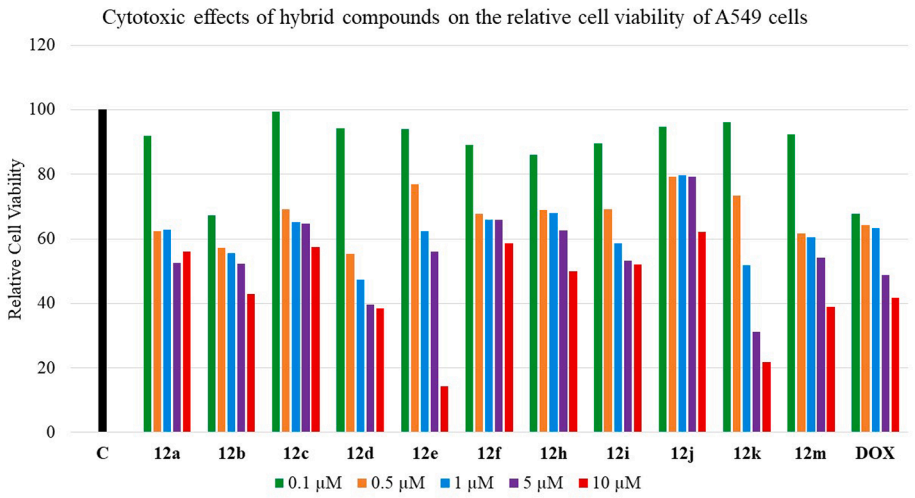


Fig. 3. Cytotoxic effects of the hybrid compounds on A549 cells.

Table 2
The IC_{50} values of the cytotoxic effects of the hybrid compounds.

Compounds	BEAS-2B (IC_{50} μM)	A549 (IC_{50} μM)	Selectivity (TD_{50}/ED_{50})
12a	1.15 ± 0.014	0.25 ± 0.009	4.6
12b	2.96 ± 0.006	0.08 ± 0.014	37.0
12c	2.79 ± 0.007	0.35 ± 0.008	7.9
12d	2.31 ± 0.010	0.31 ± 0.006	7.4
12e	5.26 ± 0.044	1.72 ± 0.010	3.0
12f	2.53 ± 0.017	0.22 ± 0.008	11.5
12h	1.12 ± 0.011	0.35 ± 0.009	3.2
12i	1.52 ± 0.014	0.28 ± 0.006	5.4
12j	0.30 ± 0.014	0.78 ± 0.014	0.3
12k	1.54 ± 0.004	0.77 ± 0.005	2.0
12m	1.01 ± 0.010	0.46 ± 0.008	2.2
Dox	0.09 ± 0.007	0.14 ± 0.009	0.6

Synthesis of acyl chloride of 3-methyl oleanolic acid (10)

A round-bottomed flask was charged with CH_2Cl_2 (250 mL) and **9b** (10 g, 21 mmol, 1 equiv.). Oxalyl chloride (3.64 mL, 43 mmol, 2 equiv.) was added in an inert atmosphere and stirred at room temperature overnight. According to the ^{13}C NMR analysis of the reaction mixture, the carboxylic acid group was completely converted into acyl chloride. The reaction solvent and excess oxalyl chloride were removed under reduced pressure which yielded the pure desired product **(10)** as a white solid in quantitative yield.

*Compound **10:*** ^1H NMR (500 MHz, $CDCl_3$) δ 5.26 (t, $J = 3.70$ Hz, 1H), 3.29 (s, 3H), 2.77 (ddd, $J = 13.60, 4.90, 1.80$ Hz, 1H), 2.59 (dd, $J = 11.70, 4.30$ Hz, 1H), 1.08 (s, 3H), 0.91 (s, 3H), 0.71 (d, $J = 8.90$ Hz, 6H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 179.76, 142.05, 123.82, 88.65, 57.56, 55.81, 47.58, 42.70, 41.72, 39.41, 38.71, 38.43, 37.05, 33.81, 32.77, 32.70, 31.27, 30.58, 28.15, 27.48, 25.87, 24.03, 23.49, 23.48, 22.01, 18.21, 16.85, 16.35, 15.37.

Synthesis of hydrazides of 3-methyl oleanolic acid (11a and 11b)

A round-bottomed flask was charged with CH_2Cl_2 (250 mL) and compound **10** (6 g, 12 mmol, 1 equiv.). Hydrazine hydrate (1 mL, 24 mmol, 2 equiv.) was added and the solution was stirred overnight at room temperature. The hydrazide dimer of 3-methyloleanolic acid formed as a minor product in this reaction. After completion of the reaction, the crude was washed with water (3x100 mL) and extracted with CH_2Cl_2 (3x150 mL). Organic layers were combined, dried over Na_2SO_4, and filtered. The solvent was removed under reduced pressure and

residue adsorbed on silica gel. The products were purified by silica gel column chromatography using an ethyl acetate and hexane mixture (1:9) eluting first the 3-methyloleanolic acid **(11b)** (yellowish-white solid, 0.45 g, 4% yield), and then the hydrazide of 3-methyloleanolic acid **(11a)** (white solid, 5.30 g, 89% yield).

*Compound **11a**:* m.p.: 128 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.06 (s, 1H), 5.25 (t, J = 3.60 Hz, 1H), 3.21 (s, 3H), 2.51 (dd, J = 11.70, 4.40 Hz, 1H), 1.01 (s, 3H), 0.82 (s, 3H), 0.76 (d, J = 5.20 Hz, 9H), 0.61 (d, J = 1.80 Hz, 6H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 179.08, 144.74, 123.43, 88.56, 57.55, 55.65, 47.54, 46.46, 45.83, 41.89, 41.43, 39.39, 38.69, 38.36, 36.96, 33.95, 32.97, 32.21, 32.13, 30.71, 28.09, 27.22, 25.83, 23.76, 23.55, 23.52, 21.97, 18.14, 16.67, 16.29, 15.31. HRMS (ESI) calculated for $C_{31}H_{52}N_2O_2$ $[M + H]^+$ 485.41070, found 485.40906 and calculated m/z $[M + Na]^+$ 507.39265 found $[M + Na]^+$: 507.39111; HPLC-PDA: λ 210 nm, MeCN:MeOH (1:1), Rt: 8.53 min, 95.5%.

*Compound **11b**:* m.p.: 327 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 9.03 (s, 1H), 5.47 (t, J = 3.60 Hz, 1H), 3.29 (s, 3H), 2.58 (td, J = 9.90, 8.40, 4.40 Hz, 2H), 1.10 (s, 3H), 0.90 (s, 3H), 0.84 (d, J = 3.20 Hz, 9H), 0.68 (d, J = 19.60 Hz, 6H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 172.55, 143.78, 124.02, 88.61, 57.54, 55.65, 47.62, 46.51, 45.83, 42.06, 41.35, 39.41, 38.70, 38.47, 36.96, 34.07, 32.91, 32.58, 32.41, 30.69, 28.16, 27.13, 25.64, 24.01, 23.70, 23.60, 22.02, 18.26, 16.96, 16.36, 15.48. HRMS (ESI) calculated m/z for $C_{62}H_{100}N_2O_4$ $[M-H]^+$ 935.76048, found 935.76129; HPLC-PDA: λ 210 nm, MeCN:MeOH (1:1), Rt: 5.01 min, 97.1%.

General procedure of synthesis of the hybrid compounds (12a-m)

A round-bottomed flask was charged with MeCN-$CHCl_3$ (50 mL) and compound **11a** (500 mg, 10 mmol, 1 equiv.) was dissolved in this solvent mixture. Corresponding benzaldehyde derivative (12 mmol, 1.25 equiv.) was added and the mixture was stirred at reflux in the presence of acetic acid (0.5 mL) in an inert atmosphere overnight. The reaction was terminated based on TLC analysis. The solvent was evaporated and the hybrid compounds (**12a-m**) were purified on silica gel using an ethyl acetate and hexane mixture (1:9).

*Compound **12a**:* This compound was synthesized using benzaldehyde (220 mg) according to the general synthesis procedure of hybrid compounds (white solid, 510 mg, 86% yield). m.p.: 175 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.96 (s, 1H), 8.03 (s, 1H), 7.65 (dd, J = 6.70, 3.00 Hz, 2H), 7.19 (s, 1H), 5.49 (d, J = 3.60 Hz, 1H), 3.29 (s, 3H), 2.59 (dd, J = 11.70, 4.30 Hz, 3H), 1.13 (s, 3H), 0.89 (s, 3H), 0.86 (s, 6H), 0.83 (s, 3H), 0.67 (d, J = 1.70 Hz, 6H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 174.81, 147.73, 145.48, 133.82, 130.36, 128.62, 127.72, 123.63, 88.59, 57.59, 55.62, 47.53, 46.77, 46.47, 42.18, 42.05, 39.54, 38.70, 38.43, 36.96, 34.04, 32.96, 32.17, 30.74, 28.11, 27.22, 25.84, 24.19, 23.76, 23.59, 22.00, 18.13, 16.99, 16.29, 15.37. HRMS (ESI) calculated m/z for $C_{38}H_{56}N_2O_2$ $[M-H]^+$ 571.42635, found 571.42755 and $[M + Na]^+$ 595.42395, found 607.40460; HPLC-PDA: λ 300 nm, MeCN:MeOH (1:1), Rt: 15.8 min, 95.3%.

*Compound **12b**:* This compound was synthesized using 4-methylbenzaldehyde (290 mg) according to the general synthesis procedure of hybrid compounds (white solid, 525 mg, 86% yield). m.p.: 178 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.93 (s, 1H), 7.97 (s, 1H), 7.55 (d, J = 7.80 Hz, 2H), 7.12 (d, J = 7.90 Hz, 2H), 5.49 (t, J = 3.60 Hz, 1H), 3.29 (s, 3H), 2.58 (td, J = 11.80, 6.10 Hz, 3H), 2.30 (s, 3H), 1.13 (s, 3H), 0.90 (s, 3H), 0.86 (s, 6H), 0.82 (s, 3H), 0.67 (d, J = 5.60 Hz, 7H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 174.66, 147.74, 145.50, 140.71, 131.05, 129.35, 127.71, 123.58, 88.58, 57.58, 55.62, 47.53, 46.77, 46.39, 42.17, 42.04, 39.53, 38.70, 38.42, 36.96, 34.05, 32.96, 32.16, 30.74, 28.11, 27.23, 25.84, 24.16, 23.75, 23.60, 22.00, 21.52, 18.13, 17.00, 16.29, 15.36. HRMS (ESI) calculated m/z for $C_{39}H_{58}N_2O_2$ $[M-H]^+$ 585.44200, found 585.44336; HPLC-PDA: λ max, MeCN:MeOH (1:1), Rt: 5.08 min, 98.3%.

*Compound **12c**:* This compound was synthesized using 4-chlorobenzaldehyde (290 mg) according to the general synthesis procedure of hybrid compounds (white solid, 560 mg, 89% yield). m.p.: 175 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 9.01 (s, 1H), 8.05 (s, 1H), 7.58 (d, J = 8.20 Hz, 2H), 7.20 (s, 0H), 5.48 (t, J = 3.60 Hz, 1H), 3.29 (s, 3H), 2.59 (dd, J = 11.70, 4.20 Hz, 2H), 1.13 (s, 3H), 0.89 (s, 3H), 0.86 (s, 6H), 0.82 (s, 3H), 0.66 (d, J = 7.90 Hz, 6H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 175.01, 146.51, 145.38, 136.24, 132.40, 131.46, 128.93, 128.82, 128.74, 123.67, 88.58, 57.59, 55.61, 47.51, 46.72, 46.51, 42.16, 41.97, 39.53, 38.70, 38.41, 36.96, 34.02, 32.94, 32.16, 32.15, 30.72, 28.11, 27.22, 25.84, 24.17, 23.74, 23.57, 22.00, 18.12, 16.96, 16.29, 15.38, 14.14. HRMS (ESI) calculated for m/z $C_{38}H_{55}{}^{35}ClN_2O_2$ $[M-H]^+$ 605.38738, found 605.38879; HPLC-PDA: λ 254 nm, MeCN:MeOH (1:1), Rt: 10.22 min, 95.4%.

*Compound **12d**:* This compound was synthesized using 4-bromobenzaldehyde (230 mg) according to the general synthesis procedure of hybrid compounds (white solid, 342 mg, 85% yield). m.p.: 178 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 9.09 (s, 1H), 8.03 (s, 1H), 7.71 – 7.33 (m, 4H), 5.47 (t, J = 3.50 Hz, 1H), 3.28 (s, 3H), 2.58 (dt, J = 9.30, 4.50 Hz, 3H), 1.12 (s, 3H), 0.89 (s, 3H), 0.81 (s, 3H), 0.66 (d, J = 11.60 Hz, 6H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 174.91, 146.44, 145.28, 132.87, 131.85, 129.01, 124.56, 123.63, 88.54, 57.57, 55.60, 47.50, 46.68, 46.47, 42.12, 41.90, 39.50, 38.69, 38.39, 36.95, 34.03, 32.96, 32.20, 32.18, 30.71, 28.12, 27.25, 25.84, 24.11, 23.73, 23.59, 21.99, 18.12, 16.98, 16.30, 15.37. HRMS (ESI) calculated m/z for $C_{38}H_{55}{}^{81}BrN_2O_2$ $[M-H]^+$ 651.33090, found 651.33673; HPLC-PDA: λ 254 nm, MeCN:MeOH (7:3), Rt: 35.81 min, 98.3%.

*Compound **12e**:* This compound was synthesized using 4-fluorobenzaldehyde (155 mg) according to the general synthesis procedure of hybrid compounds (white solid, 325 mg, 88% yield). m.p.: 175 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 9.12 (s, 1H), 8.06 (s, 1H), 7.63 (dd, J = 8.60, 5.40 Hz, 2H), 6.97 (t, J = 8.70 Hz, 2H), 5.48 (t, J = 3.60 Hz, 1H), 3.28 (s, 3H), 2.66 – 2.53 (m, 2H), 1.13 (s, 3H), 0.90 (s, 3H), 0.86 (s, 6H), 0.82 (d, J = 4.80 Hz, 3H), 0.67 (d, J = 5.10 Hz, 6H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 174.80, 164.95, 162.95, 146.50, 145.23, 130.16, 130.14, 129.53, 129.51, 129.50, 123.57, 115.82, 88.51, 57.52, 55.59, 47.50, 46.66, 46.38, 42.09, 41.84, 39.49, 38.67, 38.37, 36.94, 34.03, 32.95, 32.21, 32.17, 31.58, 30.69, 28.10, 27.25, 25.83, 24.04, 23.70, 23.58, 22.64, 21.96, 18.09, 16.98, 16.28, 15.34. HRMS (ESI) calculated m/z for $C_{38}H_{55}FN_2O_2$ $[M-H]^+$ 589.41693, found 589.41693; HPLC-PDA: λ 254 nm, MeCN:MeOH (1:1), Rt: 23.48 min, 98.8%.

*Compound **12f**:* This compound was synthesized using 3-nitrobenzaldehyde (310 mg) according to the general synthesis procedure of hybrid compounds (white solid, 600 mg, 94% yield). m.p.: 173 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 9.32 (s, 1H), 8.36 (t, J = 2.00 Hz, 1H), 8.30 (s, 1H), 8.11 (dd, J = 8.30, 2.30 Hz, 1H), 8.03 (d, J = 7.80 Hz, 1H), 5.50 (d, J = 3.60 Hz, 1H), 3.27 (s, 3H), 2.58 (dd, J = 11.70, 4.20 Hz, 1H), 1.13 (s, 3H), 0.98 – 0.84 (m, 9H), 0.81 (s, 3H), 0.66 (d, J = 2.40 Hz, 6H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 175.25, 148.47, 145.06, 144.97, 135.99, 132.74, 129.67, 124.50, 123.77, 122.40, 88.50, 57.53, 55.57, 47.49, 46.62, 46.60, 42.08, 41.77, 39.49, 38.66, 38.36, 36.94, 34.00, 32.94, 32.26, 32.18, 30.69, 28.10, 27.27, 25.84, 24.06, 23.71, 23.57, 21.96, 18.09, 16.98, 16.29, 15.37. HRMS (ESI) calculated m/z for $C_{38}H_{55}N_3O_4$ $[M-H]^+$ 616.41143, found 616.41302; HPLC-PDA: λ 254 nm, MeCN:MeOH (8:2), Rt: 39.92 min, 99.2%.

*Compound **12 g**:* This compound was synthesized using 4-nitrobenzaldehyde (310 mg) according to the general synthesis procedure of hybrid compounds (yellow solid, 595 mg, 93% yield). m.p.: 180 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 9.27 (s, 1H), 8.28 (s, 1H), 8.15 (d, J = 8.70 Hz, 2H), 7.80 (d, J = 8.50 Hz, 2H), 5.50 (d, J = 3.70 Hz, 1H), 3.29 (s, 3H), 2.60 (dd, J = 11.60, 4.50 Hz, 2H), 1.14 (s, 3H), 0.88 (d, J = 13.70 Hz, 9H), 0.83 (s, 3H), 0.67 (d, J = 3.50 Hz, 6H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 175.36, 148.49, 145.08, 144.95, 140.17, 128.10, 123.92, 123.83, 88.51, 57.56, 55.57, 47.47, 46.70, 42.11, 41.85, 39.50, 38.67, 38.37, 36.94, 33.99, 32.91, 32.23, 32.19, 32.15, 30.69, 28.10, 27.25, 25.83, 24.17, 23.72, 23.55, 21.97, 18.09, 16.95, 16.28, 15.36, 14.14. HRMS (ESI) calculated m/z for $C_{38}H_{55}N_3O_4$ $[M-H]^+$ 616.41143, found 616.41290; HPLC-PDA: λ 254 nm, MeCN:MeOH (1:1), Rt: 16.13 min, 98.1%.

*Compound **12 h**:* This compound was synthesized using 4-dimethylaminobenzaldehyde (310 mg) according to the general synthesis procedure of hybrid compounds (orange solid, 570 mg, 89% yield). m.p.:

245 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.91 – 8.76 (m, 1H), 7.83 (s, 1H), 7.52 (dt, J = 8.90, 2.40 Hz, 2H), 6.58 (dt, J = 8.80, 3.30 Hz, 2H), 5.46 (d, J = 3.70 Hz, 1H), 3.36 – 3.19 (m, 3H), 3.03 – 2.76 (m, 6H), 2.68 – 2.44 (m, 2H), 1.20 – 1.03 (m, 3H), 0.99 – 0.83 (m, 9H), 0.83 – 0.79 (m, 3H), 0.66 (dd, J = 4.8, 2.60 Hz, 6H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 174.17, 151.79, 148.31, 145.55, 129.25, 123.41, 121.37, 111.59, 88.58, 88.56, 76.89, 57.57, 55.61, 47.55, 46.80, 46.19, 42.15, 42.06, 42.04, 42.02, 40.17, 39.52, 38.69, 38.41, 36.95, 34.09, 33.00, 32.18, 30.75, 28.12, 27.24, 25.84, 24.07, 23.74, 23.63, 22.00, 18.14, 17.05, 16.30, 15.37. HRMS (ESI) calculated m/z for $C_{40}H_{61}N_3O_2$ $[M-H]^+$ 614.46855, found 614.47003; HPLC-PDA: λ 300 nm, MeCN:MeOH (1:1), Rt: 9.15 min, 96.1%.

*Compound **12i**:* This compound was synthesized using 2-methoxybenzaldehyde (280 mg) according to the general synthesis procedure of hybrid compounds (orange solid, 550 mg, 88% yield). m.p.: 205 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 9.00 (s, 1H), 8.30 (s, 1H), 7.98 (dd, J = 7.80, 1.8 Hz, 1H), 6.89 (t, J = 7.50 Hz, 1H), 6.81 (d, J = 8.30 Hz, 1H), 5.51 (t, J = 3.60 Hz, 1H), 3.79 (s, 3H), 3.29 (s, 3H), 2.59 (dd, J = 11.70, 4.30 Hz, 1H), 1.13 (s, 3H), 0.90 (s, 3H), 0.86 (s, 6H), 0.83 (s, 3H), 0.67 (d, J = 1.70 Hz, 6H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 174.51, 157.98, 145.56, 143.34, 131.59, 127.30, 123.66, 122.17, 120.93, 110.84, 88.59, 57.61, 55.63, 47.54, 46.76, 46.28, 42.16, 42.10, 39.56, 38.71, 38.43, 36.96, 34.06, 32.97, 32.17, 32.07, 30.75, 28.11, 27.22, 25.83, 24.19, 23.74, 23.60, 22.01, 18.13, 17.05, 16.29, 15.28. HRMS (ESI) calculated m/z for $C_{39}H_{58}N_2O_3$ $[M-H]^+$ 601.43692, found 601.43854; HPLC-PDA: λ 254, MeCN:MeOH (1:1), Rt: 14.58 min, 97.4%.

*Compound **12j**:* This compound was synthesized using 4-methoxybenzaldehyde (280 mg) according to the general synthesis procedure of hybrid compounds (orange solid, 540 mg, 86% yield). m.p.: 176 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.91 (d, J = 3.40 Hz, 1H), 7.95 (s, 1H), 7.66 – 7.55 (m, 2H), 6.88 – 6.77 (m, 2H), 5.47 (d, J = 3.60 Hz, 1H), 3.75 (t, J = 1.30 Hz, 3H), 3.28 (s, 3H), 2.59 (dd, J = 11.80, 4.20 Hz, 2H), 1.12 (s, 3H), 0.89 (s, 3H), 0.85 (s, 6H), 0.82 (s, 3H), 0.67 (d, J = 3.00 Hz, 6H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 174.55, 161.43, 147.48, 145.47, 129.30, 126.48, 123.52, 114.07, 88.56, 57.57, 55.61, 55.35, 47.53, 46.77, 46.33, 42.15, 42.00, 39.53, 38.69, 38.41, 36.95, 34.05, 32.97, 32.17, 30.73, 28.12, 27.23, 25.84, 24.12, 23.74, 23.60, 22.00, 18.12, 17.01, 16.29, 15.37. HRMS (ESI) calculated m/z for $C_{39}H_{58}N_2O_3$ $[M-H]^+$ 601.43692, found 601.43848; HPLC-PDA: λ max, MeCN:MeOH (1:1), Rt: 8.78 min, 99.5%.

*Compound **12 k**:* This compound was synthesized using 2,4-dimethoxybenzaldehyde (205 mg) according to the general synthesis procedure of hybrid compounds (orange solid, 285 mg, 72% yield). m.p.: 260 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.91 (s, 1H), 8.19 (s, 1H), 7.93 (d, J = 8.70 Hz, 1H), 6.44 (dd, J = 8.70, 2.30 Hz, 1H), 6.34 (d, J = 2.30 Hz, 1H), 5.56 – 5.46 (m, 1H), 3.76 (d, J = 2.80 Hz, 6H), 3.29 (s, 3H), 2.59 (dt, J = 9.40, 4.70 Hz, 1H), 1.12 (s, 3H), 0.90 (s, 3H), 0.84 (d, J = 12.30 Hz, 9H), 0.67 (d, J = 2.60 Hz, 6H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 174.30, 162.88, 159.32, 145.59, 143.34, 128.49, 123.59, 115.18, 105.46, 98.12, 88.59, 57.60, 55.63, 55.61, 55.56, 55.47, 47.54, 46.79, 46.19, 42.16, 42.11, 39.56, 38.70, 38.42, 36.96, 34.07, 32.97, 32.18, 32.08, 30.75, 28.11, 27.22, 25.82, 24.15, 23.73, 23.59, 22.01, 18.13, 17.07, 16.29, 15.28. HRMS (ESI) calculated m/z for $C_{40}H_{60}N_2O_4$ $[M-H]^+$ 631.44748, found 631.44922; HPLC-PDA: λ 290, MeCN:MeOH (1:1), Rt: 8.53 min, 95.2%.

*Compound **12 l**:* This compound was synthesized using 3,5-dimethoxybenzaldehyde (205 mg) according to the general synthesis procedure of hybrid compounds (orange solid, 280 mg, 72% yield). m.p.: 178 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 9.47 (s, 1H), 8.05 (s, 1H), 6.79 (d, J = 2.40 Hz, 2H), 6.42 (t, J = 2.30 Hz, 1H), 5.50 (t, J = 3.70 Hz, 1H), 3.73 (s, 6H), 3.32 (s, 3H), 2.63 (dd, J = 11.70, 4.30 Hz, 1H), 1.15 (s, 3H), 0.93 (s, 3H), 0.90 (d, J = 5.70 Hz, 6H), 0.85 (s, 3H), 0.70 (d, J = 13.30 Hz, 6H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 174.86, 160.85, 147.76, 145.02, 135.81, 123.44, 105.33, 103.10, 88.54, 57.49, 55.59, 55.45, 47.51, 46.57, 46.27, 42.00, 41.65, 39.42, 38.65, 38.34, 36.92, 34.03, 32.98, 32.23, 32.14, 30.67, 28.11, 27.32, 25.84, 23.82, 23.67, 23.61, 21.95, 20.99, 18.09, 16.99, 16.29, 15.30. HRMS (ESI) calculated m/z for $C_{40}H_{60}N_2O_4$ $[M-H]^+$ 631.44748, found 631.44904; HPLC-PDA: λ 254, MeCN:MeOH (1:1), Rt: 15.97 min, 98.3%.

*Compound **12 m**:* This compound was synthesized using 4-hydroxy-3-methoxybenzaldehyde (190 mg) according to the general synthesis procedure of hybrid compounds (orange solid, 290 mg, 75% yield). m.p.: 162 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.93 (s, 1H), 7.84 (s, 1H), 7.43 (d, J = 1.90 Hz, 1H), 6.89 (dd, J = 8.10, 1.80 Hz, 1H), 6.82 (d, J = 8.10 Hz, 1H), 6.02 (s, 1H), 5.48 (t, J = 3.60 Hz, 1H), 3.84 (s, 3H), 3.28 (s, 3H), 2.63 – 2.56 (m, 2H), 1.13 (s, 3H), 0.89 (s, 3H), 0.86 (s, 6H), 0.82 (s, 3H), 0.67 (d, J = 4.90 Hz, 6H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 174.69, 148.31, 147.71, 147.13, 145.50, 126.20, 123.65, 123.56, 113.95, 107.79, 88.59, 57.58, 56.16, 55.61, 47.52, 46.76, 46.33, 42.18, 42.03, 39.53, 38.70, 38.42, 36.95, 34.04, 32.96, 32.17, 30.74, 28.11, 27.24, 25.83, 24.13, 23.75, 23.59, 22.00, 18.13, 17.03, 16.29, 15.38. HRMS (ESI) calculated m/z for $C_{39}H_{58}N_2O_4$ $[M-H]^+$ 617.43183, found 617.43341; HPLC-PDA: λ 290 nm, MeCN:MeOH (1:1), Rt: 7.62 min, 98.8%.

Biological assays

Cell culture

BEAS-2B human healthy bronchial epithelial cells and A549 lung cancer cells lines were used in this study. BEAS-2B and A549 were grown in DMEM/F12 and DMEM, respectively, both supplemented with 10% FBS and 100 U/mL of penicillin–streptomycin at 37 °C in a humidified incubator with 5% CO_2. After reaching 80% confluency, the cells were detached using 0.25% trypsin-EDTA. For further experiments, cells were re-suspended in the growth medium after collection and centrifugation.

MTT assays

An MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was used to assess cell viability. Briefly, 5x10^3 cells were seeded into a flat-bottom 96-well plate with a growth medium. After 24 h incubation, it was treated with increasing doses of samples for 24 h and the assay was conducted. The absorbance values were recorded at 540 nm using a microplate reader. All the experiments were carried out in triplicates, and the results were presented as a mean ± standard deviation. The concentration-dependent graph was drawn by comparing the data for each substance whose measurement was repeated at least 3 times, and the relative % cell viability was determined [20,80–83].

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.rechem.2022.100317.

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