Synthesis and Cytotoxic Activities of Resveratrol and Valproic Acid Ester Derivatives with Anti-Bladder-Cancer Effects

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Abstract

Bladder cancer is a frequently diagnosed cancer that has caused many deaths over the past few decades, especially in men. Surgery and drugs are used for treatment, but there are no anticancer drugs that can effectively treat this condition. In this study, we synthesized a new anticancer drug with known anticancer effects. Specifically, we synthesized esters and acid anhydrides via esterification and identified a product with significant anticancer effects. The anticancer effects of (E)-5-(4-hydroxystyryl)-1,3-phenylene didodecanoate (compound **2**) on human bladder cancer cells were confirmed using an MTT assay and Annexin V staining. We found that cell viability was markedly reduced by compound **2** in bladder cancer cells at concentrations ≥ 4 mM as compared to that in control cells ($P < 0.05$). Moreover, the ratio of total apoptotic cells significantly increased at 6 mM, with low toxicity in normal cells. These results demonstrated that compound **2** may serve as a novel anticancer drug candidate for the treatment of human bladder cancer.

Introduction

Bladder cancer affects both men and women. It has the 9th highest incidence worldwide[1] and the 13th leading cause of cancer-related deaths.[1,2] Non-muscle invasive bladder cancer (NMIBC) makes up approximately 75% of all diagnosed bladder cancers. NMIBC has a high prevalence rate owing to the fact patients can survive for a long time due to its slow progression rate. Muscle-invasive bladder cancer (MIBC) accounts for up to 25% of all bladder cancers and is associated with poor prognosis. According to a report published by the National Cancer Institute (NCI), approximately 80,000 new cases of bladder cancer is diagnosed each year, and reveals an increasing trend in morbidity and mortality.[3] Bladder cancer can be treated through urethral resection,[4] radical bladder resection, and chemotherapy. [5,6] Currently, drugs such as cisplatin are commonly used for the treatment of bladder cancer. These drugs have significant anticancer effects; however, they can also cause serious side effects such as nephrotoxicity and myelosuppression.[7–9] Upon long-term use, bladder cancer cells may develop resistance to cisplatin. To solve these problems, the development of new molecules to treat bladder cancer with minimal side effects is desirable.

As high costs, development time, and high risk of attrition despite added value are some of the greatest challenges in drug development, a key strategy in drug development is to improve the nature of already existing drugs and develop them into new drugs.

Lead-compound deformation, which transforms the structure of known compounds with the desired drug characteristics, is a technique that is often applied when designing new drugs, wherein the most important aspect is the selection of a suitable lead material. Compounds contain pharmacophores, which represent the portions responsible for eliciting physiological effects when combined with receptors, and auxophores, which do not elicit physiological effects. In this study, we explored new pharmaceutical agents with pharmacologically active sites through deformation, i.e., activation, of the auxiliary action generation area after selecting a lead compound that is expected to be physiologically active.

To date, many techniques have been used in the design of new drugs. One such technique is lead-compound modification, which involves modifying the structure of a compound known to exhibit a therapeutic effect. The known compound “leads” the way to the development of other similar compounds, and is, therefore, called a lead compound. The story of aspirin provides a good example of this process. From a chemical point of view, we envision a target molecule with a low molecular weight, no toxicity, and no side effects, such as aspirin, formed from salicylic acid through an esterification reaction. To develop a molecule for the treatment of bladder cancer, we selected resveratrol, valproic acid and lauric acid as lead compounds (Fig. 1). Resveratrol, a red wine polyphenol, is a stilbenoid known for its antioxidant properties. It exists in two isomeric forms, cis- and trans-resveratrol. Trans-resveratrol was selected for this study as it is the more biologically active isomer. Resveratrol was shown not to cause side effects even when ingested at high concentrations.[10] Valproic acid has a broad spectrum of anticonvulsant activities, with an unclear mechanism of action. Recently, it was tested for the treatment of AIDS and cancer, owing to its histone-deacetylase-inhibiting effects. Lauric acid can induce apoptosis in colon cancer cells by reducing the availability of GSH through the production of ROS and regulation of the cell cycle.[11] In addition, lauric acid inhibits the proliferation of breast[12] and endometrial cancer cells.[13] It produces ROS and induces apoptosis through the phosphorylation of EGFR, ERK, and c-Jun.[14] Resveratrol is a directional stilbene-type phytoalexin found mainly in grapes, peanuts, berries, and other foods.[15] According to previous reports, resveratrol induced apoptosis in NSCLC cell lines by regulating COX-2 expression. Moreover, owing to its anticancer properties, resveratrol decreases tumor development, promotion, and progression mainly by inducing cell death through various pathways and changes in gene expression.[18–20] In this study, the biological effects of compounds on the bladder cancer cell line were investigated using methyl thiazolyl tetrazolium (MTT) and Annexin V & Dead staining assays.

Results And Discussion

First of all, we synthesized novel esters and acid anhydrides which have high anti-cancer activity with bioactive acid and alcohol. This reaction is the most basic esterification reaction and use low molecular weight molecules while has few side effects and excellent anti-cancer effect. In the first experiment, resveratrol and lauric acid were reacted, as the alcohol and acid, respectively. Three ester compounds, **1**, **2**, and **3**, were synthesized and separated using gas chromatography. In the second experiment, resveratrol and valproic acid, as the respective alcohol and acid, were reacted, not as lead compounds. Two ester compounds, compounds 4 and 5, were synthesized and successfully separated using gas chromatography. The OH positions provided the highest evidence for the synthesis of compounds as the H peaks of the OH (9.6 ppm) at the 4’-OH position completely disappeared and that of the OH (9.2 ppm) at position 3,5 remained.[21] Both aforementioned reactions were esterification reactions. In the third experiment, valproic acid and 2-acetoxybenzoic acid, acetaminophen, ibuprofen were reacted. As a result, we synthesized compounds **6**, **7**, **8**.

Second of all, to determine the effects of these compounds on 5637 cells, the cells were treated with designated concentrations of the compounds for 24 h. The cytotoxicity of the compounds was measured

using an MTT assay (Fig. 2A). Compound **2** reduced the viability of 5637 cells in a concentration-dependent manner. Furthermore, an MTT assay was used to measure the half-maximal inhibitory concentration (IC_{50}) values of the compounds using 5637 cells. As shown in Fig. 2. B, the IC_{50} of the compounds was determined at a concentration of 4.616 mM by sigmoidal curve fitting.[22] In addition, compound **2** did not affect Nuff or HDF cell viability (Fig. 2A). Subsequently, Annexin V & Dead staining assays were performed to confirm the effects of the compounds on 5637 cell death.[22] First, 5637 cells were treated with the synthesized compound at concentrations of 0, 2, 4, 6, 8, and 10 mM for 24 h. At the end of treatment, the cells were treated with Annexin V & Dead cell staining solution and analyzed using the MUSE Cell analyzer. As shown in Fig. 2C, total apoptosis increased as the concentration of compound **2** increased from 6 mM to 10 mM, with no change in the total apoptosis induced by compound **2** at concentrations less than 6 mM. Compound **2** elicited significant inhibition of cell growth at concentrations higher than 6 mM. In accordance with the cell viability test (Fig. 2A), compound **2** induced cell death in 5637 cells. In contrast, at a concentration of 6 mM, compound **2** showed no cytotoxicity in the two normal cell lines. Overall, these results suggested that compound **2** could effectively inhibit the proliferation of human bladder cancer cells without affecting normal fibroblasts. The major finding of this study was that compound **2** activated the intrinsic caspase pathway, leading to apoptosis of bladder cancer cells. Previousely, we showed that the overexpression of constitutively active form of myristoylated Akt decreased cell death of 5637 cells,[23] suggesting the critical step of Akt inactivation may be involved in imposing the anti-tumor effect of compound **2** on bladder cancer. Compound **2** may be inferred to maximize the activity effect by combining with the AKT receptor in four interactions(pi-pi stacking between aromatic rings, three ester funtional groups, non-covalent interaction involving pi system, and peripheral interaction of long straight-chain hydrocarbon), thereby increasing the activity.

Conclusion

The most basic esterification reaction was used for the synthesis of compounds in this study. When bioactive acids and alcohols with anticancer activity are synthesized and administered to humans, drug esters are hydrolyzed into the pharmacologically active alcohol and acid moieties.[22] It is known that it has a low molecular weight, few side effects, and excellent anticancer effects.[24,25] We synthesized several bioactive acids and alcohols (Table 1). Of the 9 products synthesized, only one had high anticancer activity. In this study, esterification was carried out using lauric acid and resveratrol, which has three OH groups. Previous studies have shown that lauric acid and resveratrol have anticancer effects and are useful in the management of various cancers.[10–17, 26–28]. Compound **2** is a synthetic substance, synthesized from lauric acid and resveratrol.

As evidenced by TLC after 24 h, no reaction was observed in the starting material when the reaction was initiated without using a dean-stark or sieve in various organic solvents. Thus, we added sulfuric acid as a catalyst to promote the reaction and remove water. However, because the reaction was too rapid in the presence of a catalyst, the desired product was not synthesized, and a side reaction occurred. Since esterification reactions generate water,[25,26,31] the solvent was changed to THF and reacted with a sieve to

remove water. The reaction was carried out without a catalyst. Using these conditions, the reaction was complete after 24 h.

In this study, we showed that compound **2** affects the viability of 5637 bladder cancer cells. Cell viability was found to decrease with increasing concentrations of compound **2**. Cell death was also confirmed based on apoptosis assays and dead staining. In conclusion, compound **2** was shown to elicit significant anticancer activity against bladder cancer cell lines *in vitro*.

Materials And Methods

Valproic acid, lauric acid, acetaminophen, aspirin, ibuprofen, and resveratrol were purchased from TCI (Seoul, Korea). MeOD, $CDCl_3$, and D_2O were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dimethyl sulfoxide (DMSO) was purchased from Duksan Pure Chemicals (Seoul, Korea). Dulbecco's modified Eagle medium (DMEM), Roswell Park Memorial Institute Medium (RPMI), fetal bovine serum (FBS), and penicillin/streptomycin were purchased from Capricorn Scientific GmbH (Ebsdorfergrund, Germany).

General procedures: All substrates and reagents were obtained commercially. ^{1}H-NMR spectra were measured in ppm using an Agilent MR400 (400 MHz) FT-NMR spectrometer, with $(CH_3)_4Si$ (TMS) as the internal standard and solvent. ^{13}C-NMR spectra were recorded at 100 MHz. Chemical shifts are reported with respect to the solvent (DMSO = 2.5, 3.3, and 39.52 ppm for ^{1}H- and ^{13}C-NMR). A Büchi 510 melting point apparatus, electrothermal digital melting point apparatus, and general V4 were used to determine the melting points. ^{1}C DuPont 2200. Mass spectrometry was performed using a Hewlett Packard (HP) 5890 GC/MSD [Column: HP-1 (crosslinked methyl silicone gum, 30 m × 0.25 mm × 0.3 μm); detector: 5972 A mass detector 70 eV, EI].

Reaction of resveratrol and lauric acid (Compounds **1, 2**, and **3)**: Lauric acid (0.03 mol) and resveratrol (0.01 mol) were mixed with THF (solvent). The sieve was added, and the reaction mixture was refluxed at 60°C for 24 h. After confirming the progress and completion of the reaction using TLC, the sieve was removed using vacuum filtration. The remaining sieve was removed by washing twice with THF. After eliminating the sieve by filtration, the THF in the filtrate was removed using a rotary vacuum evaporator. The products were purified using flash chromatography (EtOAc:hexane at a ratio of 2:8) to yield corresponding compounds as ivory-colored solids.

(E **)-4-(3,5-dihydroxystyryl)phenyl dodecanoate 1**: Yield: 37.2%; mp: 108 ~ 109 °C; R_f: 0.2 (TLC eluent; EtOAc: n-Hexane = 3: 7, v/v); MASS (70 eV), m/z (rel. intensity %): 410.2; ^{1}H NMR (DMSO, 400 MHz): δ; 9.24 (s, 2H), 7.57 (d, *J* = 8.6 Hz, 2H), 7.01–7.08 (m, 4H), 6.41 (d, *J* = 2.1 Hz, 2H), 6.13 (t, *J* = 2.1 Hz, 2H), 1.23 (m, 18H), 0.83 (t, *J* = 6.8 Hz, 3H); ^{13}C NMR (DMSO, 200MHz): δ; 172.257 (1C), 158.968 (2C), 150.213 (1C), 139.096 (1C), 135.145 (1C), 127.846 (2C), 127.339 (2C), 122.436 (2C), 105.132 (2C), 102.807 (1C), 33.921 (2C), 31.739 (1C), 29.425 (1C), 29.308 (1C), 29.155 (1C), 29.109 (1C), 28.822 (1C), 24.768 (1C), 22.542 (1C) 14.406 (1C); Anal. Calcd. for $C_{26}H_{34}O_4$: C, 76.06; H, 8.35; O, 15.59; Found: C, 76.02; H, 8.32; O, 15.58

(E)-5-(4-hydroxystyryl)-1,3-phenylene didodecanoate 2: Yield: 27.0%; mp: 119 ~ 120 °C; R_f: 0.3 (TLC eluent; EtOAc: n-Hexane = 3: 7, v/v); MASS (70 eV), m/z (rel. intensity %): 592.4; ^{1}H NMR (MeOD, 400 MHz): δ; 9.60 (s, 1H), 7.35 (d, *J* = 8.6 Hz, 2H), 6.99 (m, 1H), 6.88 (m, 1H), 6.80 (m, 1H), 6.77 (d, *J* = 8.6 Hz, 2H), 6.69 (s, 1H), 2.55 (m, 2H), 2.26 (m, 2H), 1.72 (m, 2H), 1.59 (m, 2H), 1.28 (m, 32H), 0.89 (t, *J* = 6.8 Hz, 6H); ^{13}C NMR (MeOD, 100 MHz): δ; 177.474 (1C), 177.329 (1C), 158.344 (1C), 153.096 (2C), 141.267 (1C), 130.250 (2C), 128.705 (1C), 125.635 (2C), 116.198 (2C), 111.191 (2C), 108.332 (1C), 34.799 (1C), 34.704 (1C) 32.777 (1C), 32.768 (1C), 30.450 (1C), 30.427 (1C), 30.313 (1C), 30.164 (1C), 30.313 (1C), 30.097 (1C), 29.948 (2C), 29.861 (2C), 25.820 (2C), 25.706 (2C), 23.436 (2C), 14.133 (2C); Anal. Calcd. for $C_{38}H_{56}O_5$: C, 76.99; H, 9.52; O, 13.49; Found: C, 77.02; H, 9.52; O, 13.48

(E)-5-(4-(dodecanoyloxy)styryl)-1,3-phenylene didodecanoate 3: Yield: 50.7%; m.p: 132–133 °C; R_f: 0.4 (TLC eluent; EtOAc: n-Hexane = 3: 7, v/v); MASS (70 eV), m/z (rel. intensity %): 774.6; ^{1}H NMR (400 MHz, $CDCl_3$): δ; 7.09 (t, *J* = 8.6 Hz, 2H), 6.88 (m, 2H), 6.77 (d, *J* = 8.6 Hz, 3H), 6.67 (d, *J* = 8.2 Hz, 2H), 2.25 (m, 6H), 1.54 (m, 6H), 1.18 (m, 48H), 1.80 (t, *J* = 6.8 Hz, 9H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ; 180.363 (3C), 156.451 (1C), 156.400 (1C), 153.653 (1C), 133.457 (2C), 129.432 (2C), 115.295 (7C), 34.150 (1C), 31.942 (1C), 29.637 (24C), 24.725(1C), 22.718 (1C), 14.132 (3C); Anal. Calcd. for $C_{50}H_{78}O_6$: C, 77.47; H, 10.14; O, 12.38; Found: C, 77.44; H, 10.12; O, 12.37

Reaction of resveratrol and valproic acid (Compounds **4** and **5)**: Valproic acid (0.03 mol) and resveratrol (0.01 mol) were mixed with THF (solvent). The sieve was added and the reaction mixture was refluxed at 60°C for 24 h. After confirming the progress and completion of the reaction by TLC, the sieve was removed using a vacuum filtration apparatus. The remaining sieve was removed by washing twice with THF. After eliminating the sieve by filtration, the THF in the filtrate was removed under vacuum. The products were purified using flash chromatography (EtOAc:hexane at a ratio of 2:8) to yield the corresponding compounds as brown solids.

(E)-4-(3-hydroxy-5-((2-propylpentanoyl)oxy)styryl)phenyl 2-propylpenoate 4: Yield: 36.1%; mp: 165 ~ 166 °C; R_f: 0.7 (TLC eluent; EtOAc: n-Hexane = 1: 9, v/v); MASS (70 eV), m/z (rel. intensity %): 480.3; ^{1}H NMR ($CDCl_3$, 400 MHz): δ; 9.20 (s, 1H), 7.38 (m, 1H), 7.36 (t, *J* = 6.3 Hz, 2H), 7.34 (m, 2H), 7.06 (t, *J* = 7.2 Hz, 4H), 1.73 (m, 4H), 1.54 (m, 4H), 1.43 (m, 8H), 1.25 (m, 2H), 0.96 (t, *J* = 7.2 Hz, 12H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ; 174.878 (2C), 150.660 (1C), 129.208 (2C), 125.505 (2C), 121.460 (2C), 100.197 (4C), 94.554 (3C), 45.234 (2C), 34.652 (4C), 20.569 (4C), 13.898 (4C); Anal. Calcd. for $C_{30}H_{40}O_5$: C, 74.97; H, 8.39; O, 16.64; Found: C, 75.00; H, 8.35; O, 16.63

(E)-5-(4-((2-propylpentanoyl)oxy)styryl)-1,3-phenylene bis(2-propylpentanoate) 5: Yield: 40.8%; mp: 184 ~ 185 °C; R_f: 0.9 (TLC eluent; EtOAc: n-Hexane = 1: 9, v/v); MASS (70 eV), m/z (rel. intensity %): 606.3; ^{1}H NMR ($CDCl_3$, 400 MHz): δ; 7.26 (t, *J* = 6.3 Hz, 4H), 6.94 (t, *J* = 7.4 Hz, 2H), 6.87 (dd, *J* = 8.5 and 0.9 Hz, 3H), 2.41 (m, 3H), 1.46 (m, 24H), 0.93 (t, *J* = 7.2 Hz, 18H); ^{13}C NMR (100 MHz, $CDCl_3$): δ; 183.048 (3C), 155.476

(3C), 129.623 (3C), 120.706 (1C), 115.280 (3C), 45.115 (3C), 34.332 (6C), 20.549 (6C), 13.970 (6C); Anal. Calcd. for $C_{38}H_{54}O_6$:C,75.21; H,8.97; O,15.82; Found: C,75.20; H,8.95; O,15.83

Synthesis of valproic acid esters using an anti-inflammatory analgesic drug (Compounds **6, 7, and 8)**: Valproic acid (0.01 mol) and acetaminophen (0.01 mol) were mixed with THF. The sieve was added and the reaction mixture was refluxed at 60°C for 24 h. After confirming the progress and completion of the reaction using TLC, the sieve was removed under vacuum. The remaining sieve was removed by washing twice with THF. After eliminating the sieve by filtration, the THF in the filtrate was removed using a vacuum rotary evaporator. The products were purified using flash chromatography (EtOAc:hexane at a ratio of 2:8) to yield the corresponding compounds, 4-acetamidophenyl 2-propylpentanoate, as brown solids.

2-acetoxybenzoic 2-propylpentanoic anhydride 6: Yield: 55.1%; mp: sticky oil, Rf: 0.6 (TLC eluent; EtOAc: n-Hexane = 1: 9, v/v); MASS (70 eV), m/z (rel. intensity %): 306.1; ^{1}H NMR (MeOD, 400 MHz): δ; 7.14 (t, *J* = 6.7 Hz, 1H), 6.79 (t, *J* = 9.0 Hz, 3H), 2.31 (t, 3H), 1.97 (s, 1H), 1.38 (m, 8H), 0.89 (t, J = 7.2 Hz, 6H); ^{13}C NMR (MeOD, 100 MHz): δ; 179.279 (1C), 171.787 (1C), 156.879 (1C), 129.048 (1C), 119.205 (4C), 114.877 (1C), 45.225 (1C), 34.525 (2C), 20.341 (2C), 13.114 (2C); Anal. Calcd. for $C_{17}H_{22}O_5$: C, 66.65; H, 7.24; O, 26.11; Found: C, 66.64; H, 7.22; O, 26.12

4-acetamidophenyl 2-propylpentanoate 7: Yield: 57.1%; mp: 89 ~ 90 ℃; R_f: 0.5 (TLC eluent; EtOAc: n-Hexane = 5: 5, v/v); MASS (70 eV), m/z (rel. intensity %): 277.2; ^{1}H NMR ($CDCl_3$, 400 MHz): δ; 7.78 (s, 1H), 7.43 (d, *J* = 7.9 Hz, 2H), 6.96 (d, *J* = 7.8 Hz, 2H), 2.60 (m, 1H), 2.10 (s, 3H), 1.53 (m, 4H), 1.42 (m, 4H), 0.95 (t, *J* = 7.2 Hz, 6H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ; 175.673 (1C), 168.718 (1C), 146.797 (1C), 135.527 (1C), 121.861 (2C), 120.920 (2C), 45.337 (1C), 34.652(2C), 22.755(1C), 20.705(2C), 14.023(2C); Anal. Calcd. for $C_{16}H_{23}NO_3$: C, 69.23; H, 8.36; N, 5.05; O, 17.30; Found: C, 69.24; H, 8.35; N, 5.06; O, 17.31

2-(4-isobutylphenyl)propanoic 2-propylpentanoic anhydride 8 : Yield: 67.3%; mp: sticky oil, Rf: 0.5 (TLC eluent EtOAc: n-Hexane = 1: 9, v/v); MASS (70 eV), m/z (rel. intensity %): 332.2; ^{1}H NMR (DMSO, 400 MHz): δ; 7.35 (t, *J* = 8.1 Hz, 2H), 7.09 (t, *J* = 7.2 Hz, 2H). 3.79 (s, 1H), 2.57 (m, 2H), 2.19 (s, 1H), 1.66 (m, 1H), 1.38 (m, 7H), 1.24 (m, 4H), 0.89 (m, 12H); ^{13}C NMR (MeOD, 100 MHz): δ; 176.254 (1C), 174.466 (1C), 140.168 (1C), 138.032 (1C), 129.946 (2C), 126.045 (2C), 63.750 (1C), 44.783 (1C), 34.598 (1C), 30.098 (2C), 24.868 (1C), 21.713 (2C), 20.440 (2C), 17.866 (2C), 13.347 (1C); Anal. Calcd. for $C_{21}H_{32}O_3$: C, 75.86; H, 9.70; O, 14.44; Found: C, 75.84; H, 9.72; O, 14.42

Cell culture

5637 (human bladder cancer cells), Nuff (human newborn foreskin fibroblasts), and HDF [human dermal fibroblasts (HDFs)] cells were purchased from the American Type Culture Collection (ATCC). 5637 cells were cultured in RPMI supplemented with 10% FBS and 1% penicillin/streptomycin (100 U/mL). Nuff and HDF cells were cultured in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin (100 U/mL). All cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2.

MTT assay

Cytotoxicity of compound **2** was evaluated using an MTT assay.[32] First, compound **2** was dissolved in DMSO. Then, it was dissolved in the free medium immediately before being used for cell treatment and was followed by 70% sonication and filtration of the material through a 0.45-μM filter (Millipore, Billerica, MA, USA). Cells were seeded into 96-well plates at a density of 1×10^4 cells/well prior to treatment. The next day, the medium was replaced with complement medium containing various concentrations of compound **2** at 37°C in a humidified CO_2 incubator. After 24 h, the medium was replaced with 100 μL of 5 mg/mL MTT solution (Sigma Chemicals, St. Louis, MO, USA) in 1XPBS PBS, and the cells were incubated for 4 h at 37°C. MTT–formazan was dissolved in 100 μL of DMSO, and the absorbance at 550 nm was measured using a microplate reader. Cell viability (%) was calculated as follows

($O.D._{550}$ of treated cells/$O.D._{550}$ of untreated cells) × 100

Annexin V and dead cell staining

Cell death and apoptosis of 5637 cells were measured using a MUSE™ Annexin V & Dead Cell kit, following the manufacturer's protocol.[33] Briefly, 5637 cells were seeded into 6-well plates at a density of 2×10^5 cells/well. After attachment, the cells were incubated with various concentrations of compound **2**. After 24 h of incubation, the cells were harvested by centrifugation and resuspended in complete media. Then, 100 μL of MUSE Annexin V & Dead cell reagents were added to each tube and incubated for 20 min at room temperature in the dark. At the end of the incubation period, cells were analyzed using a MUSE Cell Analyzer (Millipore, Billerica, MA, USA).

Statistical analyses

Each experiment was performed at least three times in all cases. Data are presented as mean ± standard deviation (SD). Statistical differences among the groups were analyzed using GraphPad Prism 5. In all of the comparisons, a level of $P < 0.05$, was considered significant.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

Not applicable.

Competing interests

Not applicable.

Funding

Not applicable.

Authors' contributions

Not applicable.

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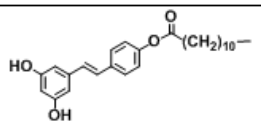
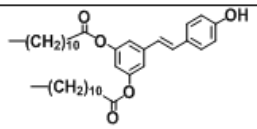
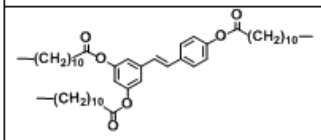
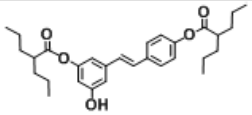
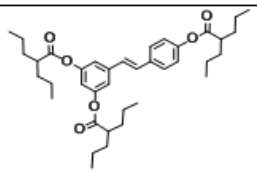
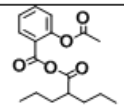
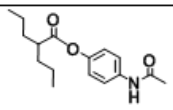
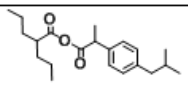
Tables

Table 1: Yields of products**1-8**

Compounds	Acid	Alcohol	Product	Yield (%) [a]
1	dodecanoic acid	resveratrol	 1	37.2
2			 2	27.0
3			 3	34.9
4	2-propyl pentanoic acid		 4	36.1
5			 5	40.8
6		2-acetoxy benzoic acid	 6	55.1
7		2-(4-isobutyl phenyl) propanoic acid	 7	77.1
8		2-(4-isobutyl phenyl) propanoic acid	 8	67.3

[a] Isolated yield.

Figures

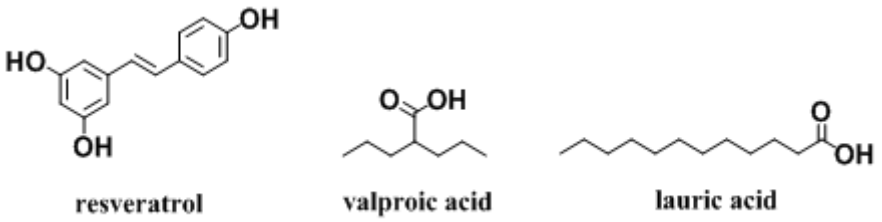


Figure 1

Structures of the selected lead compounds

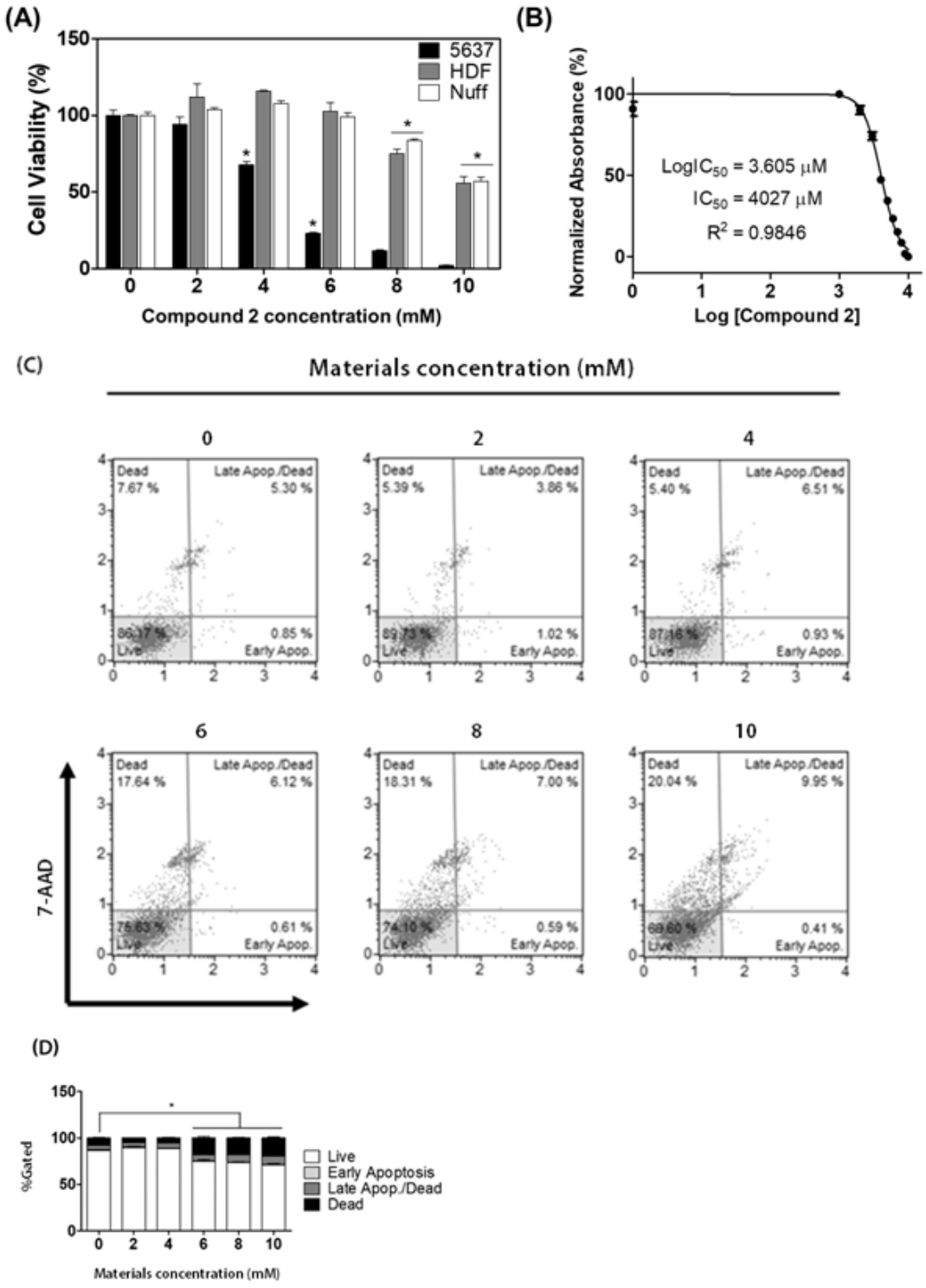


Figure 2

Cytotoxicity of compound **2**in bladder cancer cells. (A) All cells were treated with compound **2** for 24 h. (B) The altered IC_{50} of compound **2** in 5637 cells. The distribution of apoptosis in 5637 cells determined usingAnnexin V staining (C) and graph (D).Data represent the mean ± SD from independent experiments (*p< 0.05 vs controls).

Supplementary Files

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