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Dibenzoxanthenes induce apoptosis and autophagy in HeLa cells by modeling the PI3K/Akt pathway

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ABSTRACT

A new series of dibenzoxanthene derivatives **4a–4d (4a**: 1-oxo-5-bromo-11-cyano-13c-methoxy-1,13c-dihydroxyl-dibenzo[a,kl]xanthene, **4b**: 1-oxo-5-bromo-11-cyano-13c-ethoxy-1,13c-dihydroxyl-dibenzo[a,kl]xanthene, **4c:** 1-oxo-5-bromo-11-cyano-13c-propoxy-1,13c-dihydroxyl-dibenzo[a,kl]xanthene and **4d**: 1-oxo-5-bromo-11-cyano-13c-butoxy-1,13c-dihydroxyl-dibenzo[a,kl]xanthene) were synthesized and the molecular mechanisms of anti-cancer activities were investigated. These compounds showed excellent anti-tumor activity against A549, Eca-109, HeLa, HepG2 and SGC-7901 cell lines. Compounds **4a–4d** could effectively inhibit the migration and invasion of HeLa cells in wound healing and transwell assays. Compounds induced the DNA damage and arrested in cell cycle distribution at G0/G1 phase. Apoptosis induced by compounds was detected using morphological observation of nuclear changes and FITC-Annexin V/PI staining. Additionally, compounds also induced the autophagy of HeLa cells through observing AO staining and upregulated the expression of LC3II and Beclin-1 proteins. Furthermore, treatment with autophagy inhibitor 3-methyladenine induced an obvious decrease in apoptotic rate in HeLa cells. This indicated that autophagy further promoted the HeLa cells apoptosis. Compounds **4a–4d** enhanced the intracellular Ca^{2+} and ROS. Then the mitochondrial membrane potential of HeLa cells was depolarized and the cytochrome C was released from mitochondria into cytoplasm. Activities of the apoptotic factors Bcl-2, Bax, caspase-3 were measured using western blotting. After HeLa cells were exposed to compounds, the expressions of PI3K and Akt protein were decreased. Compounds exhibit anti-cancer activity via apoptosis and autophagy through inhibition of PI3K/Akt signaling pathway in HeLa cells.

1. Introduction

Apoptosis is an important process in tissue homeostasis maintenance and normal cell development of multi-cellular organism. This process is characterized by distinct morphological changes including membrane blebbing, cell shrinkage, chromatin condensation and DNA fragmentation [1]. In general, apoptosis can be initiated in two ways: the death receptor pathway and mitochondrial-dependent pathway [2]. In cancer cells, apoptosis pathways are disrupted or impacted due to which cells undergo malignant transformation. Apoptotic pathways provide a number of useful molecular targets for discovering potential chemotherapeutic agents exert anticancer effects. Many natural and synthesized products show antitumor activity by inducing mitochondrial pathway apoptosis [3–5].

Autophagy, also referred to as type II programmed cell death, was an intracellular degradation process in which double-membrane structures enclose unwanted organelles and proteins and subsequently degrade their contents upon fusion with lysosomes [6, 7]. It is well known that autophagy is a nonapoptotic programmed cell death, while autophagy and apoptosis are often simultaneously triggered by the same stimulus. Autophagy was found to have the ability to inhibit the occurrence of apoptosis, and in others, it can lead to cell death in collaboration with apoptosis in tumor therapy [8, 9]. At the molecular level, several proteins are shared in the progress of apoptosis and autophagy including Bcl-2 family proteins, FADD and some of Atg proteins. Besides the PI3k/Akt pathway is found to play vital role in both apoptosis and autophagy [10]. The PI3k/Akt signal transduction pathway is frequently deregulated in many human tumors and has attracted considerable attention as an oncology drug discovery target [11–13].

Our previous research found that dibenzoxanthenes induced cancer cells apoptosis by ROS-mediated mitochondrial pathway [14–17]. However, the role of autophagy in dibenzoxanthenes induced cell death has not been studied, and the relationship between the apoptosis and

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the autophagy is unknown yet. In this work, we synthesized four new dibenzoxanthenes **4a–4d** with Br and CN substitution simultaneously. We investigated the apoptosis and autophagy of HeLa cells induced by synthetic compounds and demonstrated the interplays between apoptosis and autophagy. Flow cytometry was used to study the intracellular ROS and mitochondrial membrane potential in HeLa cells. Further investigation was carried out for four compounds to affect the PI3K, Akt and mTOR activities.

2. Experimental

2.1. Materials and Methods

NMR spectra were recorded on a Varian spectrometer. All chemical shifts were given relative to tetramethylsilane (TMS). Electrospray mass spectra (ES-MS) were recorded on a LCQ system (Finnigan MAT, USA) using methanol as mobile phase. Microanalysis was carried out with a Perkin-Elemer 240Q elemental analyzer. TLC-analysis was performed on glass-backed plates (Merck) coated with 0.2 mm silica $60F_{254}$. Melting points were recorded using a digital melting point apparatus (IA9000 series, ThermoFischer Scientific, Rochford, U.K.) and are given without correction.

Common reagent-grade chemicals are commercially available and were used without further purification. Binaphthols and dibenzoxanthenes were prepared according to previous report [18]. Cell lines of HepG2, HeLa, A549, SGC-7901, Eca-109 and LO2 cells were purchased from American Type Culture Collection. Doubly distilled water was used to prepare buffers. 4,6-diamidino-2-phenylindole (DAPI), an ECL-Plus Kit and Cell Cycle and Apoptosis Analysis Kit were purchased from Beyotime (Shanghai, China). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H- tetrazolium bromide (MTT) was obtained from Sigma-Aldrich. The fluorescent dye 2′,7′-dichlorodihydrofluorescein diacetate (DCHF-DA) and 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine iodide (JC-1) were purchased from Roche Diagnostics (Indianapolis, IN). Polyclonal antibodies against Bim, Bcl-2 and Bcl-x were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Caspase-7 and -3 antibodies were purchased from Cell Signaling Technology (Beverly, MA).

2.2. Synthesis Procedure of Binaphthol Compound 3

A round-bottomed flask was charged with 6-cyano-2-naphthanol **1** (1 mmol) and 6-bromo-2-naphthol **2** (1 mmol), $CuCl_2$ (1 mmol), TMENDA (2 mmol) in MeOH (20 mL). The mixture was then stirred for 10 h at room temperature. The solution was with ethyl acetate (2 × 20 mL). The combined organic layers were dried over Na_2SO_4 and filtered. The solvent was removed under reduced pressure, and the residue was subjected to column chromatography on silica gel (100–200 mesh) with a mixture of EtOAc-petroleum ether (1:2, *v*/v) as eluent. The pure product **3** was obtained as yellow solid.

6-bromo-6′-cyano-1,1′-binaphthalene-2,2′-diol (3) Yield, 75%, ^{1}H NMR (300 MHz, Acetone-d_6) δ: 8.42 (s, 1H), 8.12–8.08 (m, 2H), 7.94 (d, J = 9.0 Hz, 1H), 7.52–7.33 (m, 4H), 7.29 (d, J = 9.0 Hz, 1H), 6.98 (d, J = 9.0 Hz, 1H). ^{13}C NMR (75 MHz, Acetone-d_6) δ: 157.36, 155.12, 137.15, 135.11, 133.91, 131.57, 131.13, 130.89, 130.33, 130.22, 128.88, 127.77, 127.31, 126.62, 121.38, 120.81, 120.08, 116.98, 115.51, 114.49, 106.82.

2.2.1. Synthesis of Compounds 4a–4d

To a stirred solution of $CuCl_2$ (0.350 g, 2 mmol) and ethanolamine (0.120 g, 2 mmol) in 15 mL alcohol (such as methanol, ethanol, n-propanol and n-butanol) was added binaphthol **3** (1 mmol) at 50 °C. The mixture was stirred for 4 h, the reaction was quenched with 5% $NH_3{\cdot}H_2O$ and the mixture was extracted with EtOAc. The organic extract was washed with water and dried over anhydrous Na_2SO_4. The solvent was evaporated and crude product was purified by column chromatography on silica gel (100–200 mesh) with a mixture of EtOAc-petroleum ether (1:2, *v*/v) as eluent. Yellow powder **4a–4d** were obtained.

1-oxo-5-bromo-11-cyano-13c-methoxy-1,13c-dihydroxyl-dibenzo[a,kl]xanthene (**4a**), Yield 80%, m.p. 213-214 °C, ^{1}H NMR (300 MHz, Acetone-d_6) δ: 8.47 (s, 1H), 8.23 (d, J = 9.0 Hz, 1H), 8.19 (d, J = 9.0 Hz, 1H), 7.66 (dd, J = 9.0 Hz 1.8 Hz, 1H), 7.59–7.49 (m, 3H), 7.42 (d, J = 10.2 Hz, 1H), 6.35 (d, J = 9.9 Hz, 1H), 2.80 (s, 3H). ^{13}C NMR (75 MHz, Acetone -d_6) δ:. 197.1, 153.4, 152.2, 139.1, 135.2, 134.9, 134.8, 133.4, 130.3, 129.1, 128.3, 127.2, 126.9, 124.6, 119.6, 119.4, 114.7, 107.9, 107.7, 75.0, 51.9. LC–MS, *m/z*: 417 ($[M]^+$).

1-oxo-5-bromo-11-cyano-13c-ethoxy-1,13c-dihydroxyl-dibenzo[a,kl]xanthene (**4b**), Yield 82%, m.p. 187-188 °C, ^{1}H NMR (300 MHz, Acetone-d_6) δ: 8.60 (s, 1H), 8.22 (d, J = 9.0 Hz, 1H), 8.10 (d, J = 9.0 Hz, 1H), 7.74 (dd, J = 9.0 Hz 1.8 Hz, 1H), 7.61–7.57 (m, 3H), 7.45 (d, J = 10.2 Hz, 1H), 6.41 (d, J = 9.9 Hz, 1H), 2.92–2.72 (m, 2H), 0.83 (t, J = 6.9 Hz, 3H). ^{13}C NMR (75 MHz, Acetone -d_6) δ: 196.6, 152.6, 151.4, 138.5, 134.7, 134.4 (2C), 132.7, 129.8, 128.6, 127.6, 126.7, 126.2, 123.9, 119.1, 118.8, 118.6, 114.9, 108.2, 107.2, 74.1, 59.9, 15.2. LC–MS, *m/z*: 387 ($[M\text{-}OC_2H_5]^+$).

1-oxo-5-bromo-11-cyano-13c-propoxy-1,13c-dihydroxyl-dibenzo[a,kl]xanthene (**4c**), Yield 78%, m.p. 193-194 °C, ^{1}H NMR (300 MHz, Acetone-d_6) δ: 8.59 (s, 1H), 8.21 (d, J = 9.0 Hz, 1H), 8.10 (d, J = 9.0 Hz, 1H), 7.74 (dd, J = 9.0 Hz 1.8 Hz, 1H), 7.61–7.57 (m, 3H), 7.45 (d, J = 10.2 Hz, 1H), 6.42 (d, J = 9.9 Hz, 1H), 2.92–2.72 (m, 2H), 1.78–1.73 (m, 2H), 0.58 (t, J = 6.9 Hz, 3H). ^{13}C NMR (75 MHz, Acetone -d_6) δ: 196.65, 152.56, 151.48, 138.43, 134.48, 134.41 (2C), 132.74, 129.83, 128.68, 127.63, 126.82, 126.23, 123.98, 119.07, 118.88, 118.77, 114.89, 108.21, 107.18, 74.05, 66.98, 25.08, 10.50. LC–MS, *m/z*: 387 ($[M\text{-}OC_3H_7]^+$).

1-oxo-5-bromo-11-cyano-13c-butoxy-1,13c-dihydroxyl-dibenzo[a,kl]xanthene (**4d**), Yield 74%, m.p. 195-196 °C, ^{1}H NMR (300 MHz, Acetone-d_6) δ: 8.59 (s, 1H), 8.21 (d, J = 9.0 Hz, 1H), 8.10 (d, J = 9.0 Hz, 1H), 7.74 (dd, J = 9.0 Hz 1.8 Hz, 1H), 7.61–7.57 (m, 3H), 7.45 (d, J = 10.2 Hz, 1H), 6.42 (d, J = 9.9 Hz, 1H), 2.83–2.67(m, 2H), 1.26–1.15 (m, 2H), 1.05–0.98 (m, 2H), 0.53 (t, J = 6.9 Hz, 3H). ^{13}C NMR (75 MHz, Acetone -d_6) δ: 196.65, 152.56, 151.49, 138.41, 134.78, 134.41(2C), 132.74, 129.82, 128.64, 127.63, 126.84, 126.23, 123.98, 119.06, 118.88, 118.75, 114.89, 108.20, 107.18, 74.04, 63.54, 30.86, 18.47, 13.07. LC–MS, *m/z*: 387 ($[M\text{-}OC_4H_9]^+$).

2.3. Cell Culture and Treatment

A549, Eca-109, HeLa, HepG2, SGC-7901 and LO2 cells lines were purchased from American Type Culture Collection and maintained in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 10,000 U/L penicillin, and 10 mg/L streptomycin in a humidified atmosphere of 5% CO_2 at 37 °C.

2.4. Cell Viability Assay

The effect of dibenzoxanthenes on the cell viability of A549, Eca-109, HeLa, HepG2, SGC-7901 and LO2 cells was evaluated by 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide (MTT) (Sigma Aldrich, St. Louis, MO, USA) assay. Cells were seeded at a density of 1×10^4 cells per well in a 96-well plate, and then treated with dibenzoxanthenes. After incubation for 24 h, 20 μL MTT solution (5 mg/mL), added to each well and incubated for 2 h. The viable cell number was correlated with the production of formazan, which was dissolved with dimethyl sulfoxide (DMSO), and optical density (O.D.) was measured by microplate reader (Sunrise, TECAN, Austria) at 490 nm. The IC_{50} values were determined by plotting the percentage viability versus concentration on a logarithmic graph and reading off the concentration at which 50% of cells remain viable relative to the control. Each experiment was repeated at least three times to get the mean values.