Steroids 122 (2017) 9–15

Contents lists available at ScienceDirect

Steroids

journal homepage: www.elsevier.com/locate/steroids

2-Naphthoic acid ergosterol ester, an ergosterol derivative, exhibits anti-tumor activity by promoting apoptosis and inhibiting angiogenesis

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ARTICLE INFO

Keywords:
Ergosterol monoester derivatives
Anti-tumor activity in vivo
H & E
TUNEL
Immunohistochemistry
Western blot

ABSTRACT

Phytosterol is a natural component of vegetable oil and includes ergosterol (ER) and β-sitosterol. In this study, three new ergosterol monoester derivatives were obtained from the reflux reaction with ergosterol, organic acids (furoic acid, salicylic acid, and 2-naphthoic acid), EDCI, and DMAP in dichloromethane. The chemical structures were defined by IR and NMR. On the basis of the results, 2-naphthoic acid ergosterol ester (NE) had the highest tumor inhibition rate and was selected to study anti-tumor activity and its mechanism at doses of 0.025 mmol/kg and 0.1 mmol/kg in H22-tumor bearing mice. Compared with ER, NE exhibited more stronger anti-tumor activity in vivo. Furthermore, biochemical parameters of ALT, AST, BUN, and CRE showed that NE had little toxicity to mice. NE significantly improved serum cytokine levels of IFN-γ and decreased VEGF levels. Moreover, H & E staining, TUNEL assay, immunohistochemistry, and western blotting indicated that NE exhibited anti-tumor activity in vivo by promoting apoptosis and inhibiting angiogenesis. In brief, the present study provided a method to improve ER anti-tumor activity and a reference for a new anti-tumor agent.

1. Introduction

Ergosterol (ER), which exists in yeasts, molds, and most edible and medicinal mushrooms as an important sterol, is the most important chemical raw material and the intermediates of steroid medicine [1–3]; it is used to produce progesterone, cortisone, and other drugs. ER is involved in a variety of important functions in cellular membranes. Structurally, sterols such as ER condense the fluid lipid bilayer by restraining the molecular motion of the phospholipid fatty acyl chains and increase bending rigidity and resistance against area dilation [4,5]. ER is extensively used as an index molecule of living fungal biomass [6]. ER is present in two main forms: free and esterified. The relative abundances would depend on the fungal species. Free ER plays an important role in fluidity, permeability, and integrity of the cell membrane; in addition, this molecule seems to be involved in the effects of membrane-bound proteins associated with nutrient transport and chitin synthesis [7]. However, the ER esters are stored in the hydrophobic core of cytosolic lipid particles and play a role in sterol homeostasis.

Because ER – including fat-soluble and water-soluble ER – has insolubility issues, most pharmacological studies and clinical applications of ER are limited. Therefore, modification of ER into derivatives to enhance pharmacologic activities is a viable method. Most of the modifications thus far have focused on the C3-OH of ER [7]. In the previous study, we synthesized many ER ester derivatives, and their anti-tumor activities in vitro were screened. The results of active screening showed that 2-naphthoic acid esters of ER (NE), furoic acid ergosterol ester (FE), and salicylic acid ergosterol ester (SE) had a relatively strong cytotoxic activity. On the basis of these findings, we conducted a further study on the anti-tumor activities and their mechanisms of ER and its three new monoester derivatives in vivo.

2. Methods and materials

2.1. Chemicals and reagents

ER, furoic acid, 2-naphthoic acid, salicylic acid, 4-dimethylaminopyridine (DMAP), and 1-ethyl-3-(3-dimethyllaminopropyl) carbodii-

Abbreviations: ER, ergosterol; EDCI, 1-ethyl-3-(3-dimethyllaminopropyl) carbodiimide hydrochloride; CTX, cyclophosphamide; TLC, thin-layer chromatography; IFN-γ, interferon-γ; VEGF, vascular endothelial growth factor; ALT, alanine aminotransferase; AST, aspartate transaminase; BUN, blood urea nitrogen; CRE, creatinine; TIR, tumor inhibition rate; NE, 2-naphthoic acid esters of ER; FE, furoic acid ergosterol ester; SE, salicylic acid ergosterol ester

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http://dx.doi.org/10.1016/j.steroids.2017.03.007
Received 12 December 2016; Received in revised form 23 March 2017; Accepted 27 March 2017
Available online 01 April 2017
0039-128X/

mide hydrochloride (EDCI) were purchased from Sigma-Aldrich (Shanghai) Trading Co., Ltd (Shanghai, China). Cyclophosphamide (CTX) for injection was purchased from Shanghai Huili Biotechnology Co., Ltd. TLC plates were obtained from Qingdao Haiyang Chemical Co., Ltd., and silica gel (200–300 mesh, Qingdao Haiyang Chemical Co., Ltd.) was used for column chromatography. Hematoxylin and eosin (H & E) dye kits were obtained from Nanjing Jiancheng Bioengineering Research Institute (Nanjing, China). TUNEL assay was performed in situ by using the apoptosis detection kit (Roche, Branchburg, NJ, USA) and the DAB detection kit. Interferon-γ (IFN-γ) and vascular endothelial growth factor (VEGF) ELISA kits were purchased from American R & D Co., Ltd. (Minneapolis, MN, USA). ALT (alanine aminotransferase), AST (aspartate transaminase), BUN (blood urea nitrogen), and CRE (creatinine) reagent kits were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Rabbit monoclonal anti-Bax, anti-Bcl-2, and anti-VEGF antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). All other chemicals were of analytical grade from Beijing Chemical Factory.

2.2. Synthesis of compounds

To a solution of 2-naphthoic acid (0.3 mmol) and EDCI (0.4 mmol) in 5 mL dichloromethane stired for 10 min, we added a solution of 0.2 mmol of ER and DMAP (0.2 mmol) in 5 mL dichloromethane. After the solution was heated to reflux for 8 h, the precipitate was removed by filtration, and the solvent was evaporated under reduced pressure. The residue was purified by silica gel chromatography and eluted with petroleum ether/ethyl acetate (5:1, v/v) to yield the product as a light white solid (89.8 mg). The purity of the product was determined as 97.3% by HPLC, and the structure was established by IR and NMR analysis. The other series of ER esters were prepared by a similar procedure.

2.3. Structural determination

The molecular structures of ER esters were identified by ^{1}H NMR and ^{13}C NMR analysis recorded on Varian Mercury 300 MHz NMR spectrometer equipped with a superconducting magnet (Oxford Instruments Ltd., alo Alto, CA, USA). $CDCl_3$ was used as a solvent to dissolve samples, and tetramethylsilane was used as the internal standard for NMR analysis. FTIR analysis of ER esters was performed using a WGH-30A double-beam infrared spectrophotometer (Gangdong Sci & Tech. development Co., Ltd. Tianjin, China).

2.3.1. NE

Yield 79.0%, white powder, $C_{39}H_{50}O_2$. IR: 3083, 2985–2875, 1725, 1603, 1582, 1505, 1383, 1371 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$) δppm: 8.618 (*s*, 1H, 1″-H), 8.102 (*dd*, 1H, *J* = 1.5,8.7 Hz, 8″-H), 7.973 (*dd*, 1H, *J* = 1.2,8.7 Hz, 5″-H), 7.890 (*d*, 2H, *J* = 8.7 Hz, 3″,4″-H), 7.611 (*ddd*, 1H, *J* = 1.2,8.7,14.4 Hz, 6″-H), 7.588 (*ddd*, 1H, *J* = 1.5,8.7,14.4 Hz, 7″-H), 5.649 (*m*, 1H, 6-H), 5.427 (*m*, 1H, 7-H), 5.240 (*dd*, 1H, *J* = 4.2,7.2 Hz, 22-H), 5.221 (*dd*, 1H, *J* = 4.2,7.2 Hz, 23-H), 5.043 (*m*, 1H, 3-H), 1.075 (*d*, 3H, *J* = 6.6 Hz, 21-H), 1.031 (*s*, 3H, 18-H), 0.956 (*d*, 3H, *J* = 6.6 Hz, 28-H), 0.877 (*d*, 3H, *J* = 6.9 Hz, 26-H), 0.862 (*d*, 3H, *J* = 6.6 Hz, 27-H), 0.657 (*s*, 3H, 19-H), 2.754 ~ 0.573 (others); ^{13}C NMR (75 MHz, $CDCl_3$) δppm: 165.05 (C1′), 140.47 (C8), 137.45 (C5), 134.52 (C22), 134.41 (C4a″), 131.44 (C8a″), 130.91 (C23), 129.86 (C1″), 128.26 (C8″), 127.06 (C6″), 126.98 (C4″), 126.93 (C5″), 126.68 (C2″), 125.50 (C7″), 124.24 (C3″), 119.30 (C6), 115.31 (C7), 72.50 (C3), 54.64 (C17), 53.47 (C14), 45.00 (C9), 41.78 (C13,C24), 39.42 (C20), 37.98 (C12), 36.94 (C1), 36.11 (C10), 35.78 (C4), 32.04 (C26), 27.26 (C2), 27.21 (C16), 21.96 (C15), 20.09 (C11), 19.99 (C26), 18.94 (C27), 18.63 (C21), 16.59 (C28), 15.19 (C18), 11.02 (C19).

2.3.2. FE

Yield 85.2%, white powder, $C_{33}H_{46}O_3$. IR: 3052, 2972–2884, 1730, 1652, 1384, 1370 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$) δppm: 7.504 (*dd*, 1H, *J* = 0.9,1.8 Hz, 5″-H), 7.111 (*dd*, 1H, *J* = 0.9,3.6 Hz, 3″-H), 6.441 (*dd*, 1H, *J* = 1.8,3.6 Hz, 4″-H), 5.542 (*m*, 1H, 6-H), 5.334 (*m*, 1H, 7-H), 5.151 (*dd*, 1H, *J* = 4.2,7.2 Hz, 22-H), 5.137 (*dd*, 1H, *J* = 4.2,7.2 Hz, 23-H), 4.883 (*m*, 1H, 3-H), 0.982 (*d*, 3H, *J* = 6.6 Hz, 21-CH_3), 0.916 (*s*, 3H, 18-H), 0.860 (*d*, 3H, *J* = 6.9 Hz, 28-H), 0.781 (*d*, 3H, *J* = 6.9 Hz, 26-H), 0.766 (*d*, 3H, *J* = 6.9 Hz, 27-H), 0.565 (*s*, 3H, 19-H), 2.588–0.479; ^{13}C NMR (75 MHz, $CDCl_3$) δppm: 157.18 (C1′), 145.07 (C5″), 144.06 (C2″), 140. 60 (C8), 137.28 (C5), 134.54 (C22), 130.96 (C23), 119.38 (C6), 116.68 (C3″), 115.28 (C7), 110.74 (C4″), 72.53 (C3), 54.69 (C17), 53.50 (C14), 45.02 (C9), 41.80 (C13,24), 39.42 (C20), 38.00 (C12), 36.90 (C1), 36.10 (C4), 35.67 (C10), 32.07 (C25), 27.26 (C2), 27.15 (C16), 21.97 (C15), 20.09 (C11), 20.01 (C26), 18.93 (C27), 18.63 (C21), 16.59 (C28), 15.16 (C18), 11.04 (C19).

2.3.3. SE

Yield 87.9%, light yellow powder, $C_{35}H_{48}O_3$. IR: 3440, 3080, 2974–2875, 1725, 1674, 1604, 1582, 1382, 1370 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$) δppm: 10.904 (*s*, 1H, 2″-OH), 7.871 (*dd*, 1H, *J* = 1.5,7.8 Hz, 6″-H), 7.477 (*dt*, 1H, *J* = 1.8,7.8 Hz, 4″-H), 6.990 (*d*, 1H, *J* = 7.8 Hz, 3″-H), 6.904 (*dt*, 1H, *J* = 0.9,7.8 Hz, 5″-H), 5.637 (*m*, 1H, 6-H), 5.421 (*m*, 1H, 7-H), 5.281 (*dd*, 1H, *J* = 4.2,7.2 Hz, 22-H), 5.140 (*dd*, 1H, *J* = 4.2,7.2 Hz, 23-H), 5.029 (*m*, 1H, 3-H), 1.062 (*d*, 3H, *J* = 6.6 Hz, 21-CH_3), 1.009 (*s*, 3H, 18-H), 0.940 (*d*, 3H, *J* = 6.9 Hz, 28-H), 0.860 (*d*, 3H, *J* = 6.6 Hz, 26-H), 0.845 (*d*, 3H, *J* = 6.6 Hz, 27-H), 0.649 (*s*, 3H, 19-H), 2.672–0.562. ^{13}C NMR (75 MHz, $CDCl_3$) δppm: 168.62 (C1′), 160.70 (C2″), 140.71 (C8), 136.99 (C5), 134.51 (C22,C4″), 130.98 (C23), 128.86 (C6″), 119.56 (C5″), 117.98 (C6), 116.51 (C3″), 115.27 (C7), 111.80 (C1″), 73.08 (C3), 54.69 (C17), 53. 52 (C14), 45.02 (C9), 41.80 (C13,24), 39.40 (C20), 37.99 (C12), 36.85 (C1), 36.11 (C10), 35.60 (C4), 32.07(C25), 27.27 (C2), 27.11(C16), 21.98 (C15), 20.10 (C11), 20.03 (C26), 18.94 (C27), 18.64 (C21), 16.60 (C28), 15.19 (C18), 11.06 (C19).

2.4. Antitumor activity in vivo

2.4.1. Animals and cell lines

Specific Pathogen Free grade, male ICR mice (18–22 g) were purchased from Changchun Yisi experimental Animal Technology Co., Ltd. The mice were kept in the laboratory under aseptic condition: (23 ± 2 °C, 55 ± 5% humidity) on a 12-h light/dark cycle. The mice were provided with a standard pellet diet and water *ad libitum* during the experimental period. Mouse H22-hepatoma cell line was purchased from the Institute of Biochemistry and Cell Biology, SIBS, CAS, Shanghai, China. Murine H22 cells were maintained in the ascitic form by sequential passages into the peritoneal cavities of male ICR mice, as previously described [8].

2.4.2. Tumor-bearing mice model and treatments

A tumor cell suspension of murine H22-hepatoma cells was prepared with physiological saline at a concentration of 1×10^5 cells/mL. The mice were inoculated in the subcutaneous right forelimb armpit with the tumor cell suspension (0.2 mL for each mice) to establish the tumor-bearing mice model. After 24 h, the tumor-bearing mice were randomly divided into five groups, with 8 mice in each group. The mice were intraperitoneally injected with 0.1 mmol/kg body weight of ER, FE, SE, and NE dissolved in soybean oil, respectively (treatment groups), and equal volumes of soybean oil (normal and model groups) 1 time/d for 14 d. The tumor-bearing mice were observed daily and weighed every alternate day. All mice were sacrificed by cervical dislocation. Tumor tissues were excised and weighted. The index of tumor inhibition rate (TIR) was calculated as 100% × (average tumor weight of the control group − average tumor weight of the treatment group)/average tumor weight of the control