European Journal of Pharmacology 749 (2015) 107–114

Contents lists available at ScienceDirect

European Journal of Pharmacology

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

Molecular and cellular pharmacology

The synergistic antitumor effects of all-trans retinoic acid and C-phycocyanin on the lung cancer A549 cells *in vitro* and *in vivo*

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ARTICLE INFO

Article history:
Received 10 September 2014
Received in revised form
14 January 2015
Accepted 15 January 2015
Available online 21 January 2015

Keywords:
All-trans retinoic acid
C-phycocyanin
A-549 cancer cells
Apoptosis
Cell proliferation

ABSTRACT

The anticancer effects and mechanism of all-trans retinoic acid (ATRA), C-phycocyanin (C-PC) or ATRA+C-PC on the growth of A549 cells were studied in *in vitro* and *in vivo* experiments. The effects of C-PC and ATRA on the growth of A549 cells were determined. The expression of CDK-4 and caspase-3, and the cellular apoptosis levels were detected. The tumor model was established by subcutaneous injection of A549 cells to the left axilla of the NU/NU mice. The weights of tumor and the spleen were tested. The viabilities of T-cells and spleen cells, TNF levels, the expression of Bcl-2 protein and Cyclin D1 gene were examined. Results showed both C-PC and ATRA could inhibit the growth of tumor cells *in vivo* and *in vitro*. ATRA+C-PC cooperatively showed a higher antitumor activity. The dosage of ATRA was reduced when it was administered with C-PC together, and the toxicity was reduced as well. ATRA+C-PC could decrease CDK-4 but increase caspase-3 protein expression level and induce cell apoptosis. ATRA alone could lower the activities of T lymphocytes and spleen weights, but the combination with C-PC could effectively promote viability of T cells and spleen. C-PC+ATRA could up-regulate TNF, and down-regulate Bcl-2 and Cyclin D1 gene. The combination might inhibit tumor growth by inhibiting the progress of cell cycle, inducing cell apoptosis and enhancing the body immunity.

1. Introduction

Cancer is a class of diseases characterized by out-of-control cell growth and it has become one of the leading causes of death in the world. Cancer symptoms are quite varied and depend on where the cancer is located, where it spreads, and how big the tumor grows. The clinical treatment of cancer includes surgery and chemotherapy with severe disadvantage of drug resistance and toxicity (Pathan et al., 2010; Zhang et al., 2011), so it is necessary to find some novel therapies to reduce the toxicity of drugs.

Spirulina platensis has been widely used for 100 years as an excellent nutrient supplement for human beings. C-phycocyanin (C-PC) is extracted from *S. platensis* and is a water-soluble protein pigment. This protein consists of α and β subunits and its natural existing form is trimeric aggregation $(\alpha\beta)_3$ (Ducret et al., 1996; Marquardt et al., 1997). C-PC is a famous health food because of its antioxidation (Thangam et al., 2013), antivirus (Cherng et al., 2007), anti-tumor (Subhashini et al., 2004; Li et al., 2010) and radical scavenging effects (Bhat and Madyastha, 2000).

All-trans retinoic acid, a derivative of vitamin A, exhibits great effects on cell differentiation (Chen et al., 2009; Congleton et al., 2011; Hung et al., 2008). In acute promyelocytic leukemia (APL), ATRA is a potent inducer of APL cell differentiation. However, the therapeutic use of this drug is limited by its toxicity (Siddikuzzaman and Grace, 2012). To solve this problem, several chemotherapeutic drugs such as arsenic trioxide (As_2O_3) (Zhao et al., 2001) and fludarabine (Montillo et al., 2009) were used in combination with ATRA.

For the same purpose, we focused on investigating the effects of ATRA, C-PC or ATRA+C-PC on the growth, cell cycle distribution and apoptosis of A549 cells in *in vitro* and *in vivo* studies, further revealing the anticancer mechanism of the drug combination.

2. Materials and methods

2.1. Materials

A549 cells were kindly provided by the Affiliated Hospital of Medical College of Qingdao University. *S. platensis* tablets were purchased from the Ocean University of China. All-trans retinoic acid was

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http://dx.doi.org/10.1016/j.ejphar.2015.01.009
0014-2999/

purchased from Sigma-Aldrich Group. Methyl thiazolyl tetrazolium (MTT) was purchased from Beijing solarbio science & technology. Rabbit anti-human Cyclin D1 monoclonal antibody was purchased from Boster Biological Engineering Co., Ltd., and biotinylated rabbit anti-human bcl-2 monoclonal antibody was from Santa Co., Ltd; RNAiso PLUS kit and PrimeScript RT-PCR kit were from Takara. Cdk 4 *in situ* hybridization kit, *in situ* cell apoptosis detection kit, DAB staining kit, immunofluorescence staining kit, and immunohistochemistry staining kit were purchased from Boster Biological Engineering Co., Ltd.

2.2. Cell culture

A549 cells were incubated in RPMI 1640 supplemented with 10% fetal calf serum and cultured at 37 °C in an atmosphere of 5% CO_2. Exponentially growing cells were used in all experiments.

2.3. Drugs

C-phycocyanin was extracted from *S. platensis* (Li et al., 2005) and was dissolved in phosphate buffer saline (PBS) and stored at −20 °C. All-trans retinoic acid was kept at a concentration of 10 mM in ethanol at −20 °C in dark place and diluted before use.

2.4. Cell proliferation and clonogenic assay

MTT assay was used to measure the effect of C-PC and ATRA on the proliferation of A549 cells. The cells (1×10^4 cells/well) were seeded into a 96-well plate in 100 μl medium and cultured overnight. Twenty-four hours later, six different concentrations of drugs were added to the plates at a volume of 100 μl per well and the plates were incubated for 48 h with the drugs. Then, 20 μl of MTT was added to each well and incubated for 4 h at 37 °C. The medium was carefully discarded. The formazan crystals were dissolved in 100 μl dimethylsulfoxide (DMSO) and the absorbance was read at 490 nm by a spectrophotometer (Tolis et al., 1999). Absorbance values were expressed as a percentage of drug treated group and untreated control group. The half-maximal inhibitory concentration (IC_{50}) of each drug was calculated. The analytic results of Calcusyn software showed half of the IC_{50} values of C-PC and ATRA were used as the synergy drug dose in the following experiments including 2.6, 2.7 and 2.8.

2.5. Groups of in vitro experiment and analysis of combination index

A549 cells were divided into four groups: (1) control group: the cells were not treated with any drug. (2) C-PC treated group: the cells were treated with C-PC alone. (3) ATRA treated group: the cells were treated with ATRA alone. (4) C-PC+ATRA combined group: the cells were treated simultaneously with C-PC and ATRA. For the combined treatment group, the combination index (CI) between two drugs was calculated. CI values of less than, equal to, and more than 1 indicate synergy, additivity, and antagonism, respectively (Liu et al., 2013; Rodea-Palomares et al., 2010).

2.6. Detection of CDK4 mRNA by in situ hybridization (ISH)

A 96 bp of oligonucleotide probe for human CDK4 mRNA (5′-ACCTT TAACC CACAT AAGCG AATCT CTGCC TT-3′, labeled with digoxin at 5′ end) was synthesized. The cells were seeded on the poly-L-lysine coated coverslip in a 6-well plate, treated with the drugs for 48 h and then fixed in freshly prepared paraformaldehyde solution (4% in PBS, pH 7.4) for 30 min at room temperature. Endogenous peroxidase was inactivated by 3% H_2O_2. Binding sites were exposed by digestion with pepsin (diluted by 3% citric acid) for 2 min at 37 °C. Prehybridization was carried out at 38 °C for 4 h and hybridization was at 38 °C for 12 h. Then, the coverslips were washed sequentially by 2 × SSC for 5 min, 0.5 × SSC for 15 min and 0.2 × SSC for 15 min at 37 °C. Cells were incubated in blocking buffer for 30 min and in mouse anti-digoxin IgG for 60 min at 37 °C. After washed by PBS, the coverslips were immersed in streptavidin–biotin complex (SABC) for 20 min and in biotinylated peroxidase for 30 min at 37 °C. Finally, the coverslips were stained with diaminobenzidine (DAB) and observed under a light microscope. Integrated optical density (IOD) of each group was calculated by Image-Pro Plus (IPP) and used as index for CDK4 mRNA levels.

2.7. Apoptosis determination by TUNEL assay

A549 cells were washed with PBS and fixed in 4% paraformaldehyde. DNA breaks were labeled sequentially by TdT, bromodeoxyuridine triphosphate (BrdUTP) and DIG-labeled anti-BrdU antibody. Then the cells were incubated in blocking buffer, biotinylated anti-digoxin IgG, SABC and DAB in turn. IOD of each group was calculated by IPP and used as an index of apoptosis levels.

2.8. Determination of caspase-3 protein by Western blot

A549 cells were harvested, washed twice with cold PBS, lysed for 2 h on ice, and centrifuged at 4 °C to remove insoluble materials. The lysate supernatant was separated using SDS-PAGE gel electrophoresis and transferred to polyvinylidene fluoride (PVDF) membranes. Subsequently, the membrane was blocked with TBS (containing 5% skim milk powder, pH 7.4) and then incubated overnight in rabbit anti-human caspase-3 and rabbit anti-human β-actin antibodies, and then in HRP-conjugated goat anti-rabbit secondary antibody for 2 h. Finally, the caspase-3 protein was detected by DAB. The bands were scanned and analyzed by Image J software. Caspase-3 levels were normalized with respect to β-actin levels and grayscale ratio of caspase-3/β-actin was calculated.

2.9. The construction of mice model with tumor and grouping

A total of 40 adult NU/NU mice (20 males and 20 females, 20–22 g) were used for the study. The mouse tumor models were set up by subcutaneous injection of 2×10^6 A549 cells near the armpit area. Fifteen days later, the mice were randomly divided into four groups: control group, ATRA treatment group which was treated with 0.2 ml ATRA (10 mM), C-PC treatment group which was given 0.2 ml C-PC (320 mg/ml), and C-PC+ATRA treatment group receiving 0.2 ml C-PC (320 mg/ml) and 0.2 ml ATRA (10 mM) at the same time. These agents were injected into the area of tumors and the duration of drug treatment was 10 days. Two days after drug withdrawal, the mice were executed, and then tumors and spleens were picked out. All of the mice survived. All studies were approved by the Qingdao University institutional animal care and use committee. Animals were housed under standard conditions with *ad libitum* food and water and a 12:12 light: dark cycle at the Qingdao University facilities.

2.10. The effects of single drug and combination therapy of two drugs on tumor

The tumor weights of the four groups of mice were compared.

2.11. The effects of single drug and combination therapy of two drugs on immune organ

The spleen weights of the four groups of mice were compared.