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SCIENCE DIRECT
Toxicology and Applied Pharmacology 210 (2006) 55 – 62
Toxicology and Applied Pharmacology
www.elsevier.com/locate/ytaap

Enhancement of esculetin on Taxol-induced apoptosis in human hepatoma HepG2 cells

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Received 22 March 2005; revised 14 June 2005; accepted 21 June 2005
Available online 26 July 2005

Abstract

The potential use of low dose chemotherapy has been appealing since lower dosages are more attainable during cancer therapy and cause less toxicity in patients. Combination therapy of Taxol, a promising frontline chemotherapy agent, with natural anti-tumor agents that are considerably less toxic with a capability of activating additional apoptotic signals or inhibiting survival signals may provide a rational molecular basis for novel chemotherapeutic strategies. Esculetin, a well-known lipoxygenase inhibitor, showed an inhibitory effect on the cell cycle progression of HL-60 cells in our previous study. In this report, the effects of a concomitant administration of esculetin and Taxol were investigated in human hepatoma HepG2 cells. Firstly, esculetin alone could exert an antiproliferation effect together with an inhibitory effect on the activation of ERKs and p38 MAPK. As compared to the treatment with Taxol only, a co-administration with esculetin and Taxol could result in a further enhancement of apoptosis as revealed by DNA fragmentation assay and Annexin-V-based assay. Meanwhile, immunoblotting analysis also showed that the co-administration of esculetin and Taxol could increase the expression of Bax and the cytosolic release of cytochrome C and enhance the expression of Fas and Fas ligand while the activation of caspase-8 and caspase-3 was also increased. Finally, the ERK cascade was proven to be involved in the enhancement of esculetin on the Taxol-induced apoptosis.

Keywords: Esculetin; Taxol; HepG2 cells; Apoptosis

Introduction

Esculetin, a coumarin derivative contained in various plants such as *Artemisia scoparia*, *Artemisia capillaries* (Compositae), *Ceratostigma willmottianum*, (Plumbaginaceae), and in the leaves of *Citrus limonia* (Rutaceae), are commonly utilized as folk medicines (Chang et al., 1996; Yue and Xu, 1997). This compound could exert an inhibitory action on various biological aspects including the activities of lipoxygenase (Kemal et al., 1987; Neichi et al., 1983) and xanthine oxidase (Egan et al., 1990), platelet aggregation (Okada et al., 1995), *N*-methyl-*N*-nitrosourea-induced mammary carcinogenesis in rats (Matsunaga et al., 1998), and benzo[a]pyrene plus 4-(methylnitrosoamino)-1-(3-pyridyl)-1-butanone-induced lung tumorigenesis in A/J mice (Hecht et al., 1999). In addition, it has been proven that esculetin has an antioxidant activity (Lin et al., 2000; Pyay et al., 1992) and an inhibitory effect on the growth of human breast cancer cells and vascular smooth muscle cells (Noguchi et al., 1995; Pan et al., 2003), as well as a synergistic effect with retinoic acid on the differentiation of human leukemia cells (Hofmanova et al., 1998). Furthermore, our previous studies has demonstrated the anti-tumor potential of esculetin associated with the induction of apoptosis (Chu et al., 2001) and the inhibition of cell cycle progression in human leukemia HL-60 cells (Wang et al., 2002).

Abbreviations: DAPI, 4,6-diamidino-2-phenylindole; DMSO, dimethyl sulfoxide; ERK, extracelluar signal-regulated kinase; JNK, c-Jun NH_2-terminal kinase; p38 MAPK, p38 mitogen-activated protein kinase.

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doi:10.1016/j.taap.2005.06.020

Taxol is a widely used cancer chemotherapeutic drug that exhibits clinical activity in a range of human malignancies (Arbuck et al., 1993; Rowinsky and Donehower, 1995). The biological activity of Taxol is based on its ability to stabilize microtubules inducing microtubule bundling (Horwitz, 1992; Torres and Horwitz, 1998). Treatment of cells with Taxol disrupts the formation of normal spindles at metaphases, leading to an arrest of the cells in the G2/M phase of the cell cycle and eventually to an apoptotic cell death (Bhalla et al., 1993). In addition to the well-documented effects on microtubules, a number of reports have described the activation of components of the mitogen-activated protein kinase (MAPK) pathway in response to Taxol treatment. These studies suggested that the activation of various family members is variable and depend on the cell model (Lieu et al., 1998; Shtil et al., 1999; Stone and Chambers, 2000). For example, Taxol activated JNK in human ovarian cancer OVCA 420 cells, and the activation of JNK contributes to tumor cell apoptosis (Wang et al., 1999). However, it was reported that both ERK and p38 MAPK cascades in MCF7 cells are essential for apoptotic response to Taxol-induced cell death (Bacus et al., 2001). Hence, the relevance of MAPK activation to Taxol-induced death has not been fully resolved. Therefore, in this report, we evaluated the impact of esculetin, as well as the involvement of MAPKs, on Taxol-induced apoptosis of HepG2 cell.

Since hepatocellular carcinoma remains a major challenging clinical problem in many parts of the world especially in Eastern Asia and Southern Africa, it is imperative to develop more effective chemotherapy strategies. The use of several anti-tumor agents in combination is under intense evaluation, and numerous co-administrations of Taxol and less conventional agents have been investigated (Carré et al., 2002; Mcdaid and Horwitz, 2001; Yu et al., 2001). This study is the first to investigate the co-administration of esculetin and Taxol and aimed to provide a rational molecular basis for novel chemotherapeutic strategies.

Materials and methods

Cell culture. Human hepatoma HepG2 cells were grown in the Dulbecco's modified Eagle's medium (GIBCO BRL, Grand Island, NY) supplemented with 10% fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine. The cells were plated at a density of $2 \times 10^4/cm^2$ into tissue-culture dishes and grown at 37 °C in a humidified incubator with 5% CO_2 atmosphere.

Trypan blue exclusion assay. Cells (1×10^5 cells/well) were grown in 6-well culture plates for 24 h and exposed to different concentrations of esculetin (25, 50, and 100 μM) for an additional 24 h. Floating and adhering cells were collected and stained with 0.4% of trypan blue for 5 min at room temperature before they were examined under the microscope. The number of viable cells was determined by trypan blue exclusion, and the results were expressed as the % of control.

Determination of DNA fragmentation. Both detached and attached cells were harvested by scraping and centrifugation. After being washed with PBS (with 1 mM $ZnCl_2$), the cells were resuspended in 0.5 ml lysis buffer (0.5% Triton X-100, 20 mM EDTA, and 5 mM Tris; pH 8.0) for 45 min. Fragmented DNA in the supernatant fraction after a centrifugation at 14,000 rpm was extracted twice with phenol:chloroform:isoamyl alcohol (25:24:1, v/v/v) and once with chloroform, and then it was precipitated with ethanol and 5 M NaCl at −20 °C overnight. The DNA pellet was washed once with 70% ethanol, resuspended in Tris–EDTA buffer (pH 8.0), and treated with 100 μg/ml RNase A for 2 h at 56 °C. After the quantitative analysis of DNA content by spectrophotometry (260 nm), an equal amount of DNA was electrophoresed in the horizontal agarose gel (1.5%) performing at 1.5 V/cm for 3 h. The DNA present in the gel was visualized under UV light after it was stained with ethidium bromide (0.5 mg/ml).

Detection of apoptosis by Annexin-V FITC staining. Briefly, HepG2 cells were treated in one of three different manners; that is, they were treated with Taxol or esculetin alone or they were first treated with esculetin and then with Taxol later. The three treatments all lasted for 18 h, and after being harvested, cells were resuspended ($2–3 \times 10^6$ cells/ml) in Annexin-V binding buffer containing 10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$. Aliquots of cells (100 μl/tube) were incubated with 5 μl of Annexin-V fluorescein isothiocyanate (FITC), mixed, and incubated for 15 min at room temperature in the dark. Propidium iodide (PI) at a concentration of 5 μg/ml was added to distinguish the necrotic cells. The apoptotic cells (V^+/PI^-) were measured by the fluorescence-activated cell sorter analysis in a FACS analyzer (Becton Dickinson). The data represented three independent experiments.

Preparation of total cell extracts and immunoblots analysis. To prepare the whole-cell extract, cells were washed with PBS plus zinc ion (1 mM) and suspended in a lysis buffer (50 mM Tris, 5 mM EDTA, 150 mM NaCl, 1% NP 40, 0.5% deoxycholic acid, 1 mM sodium orthovanadate, 81 μg/ml aprotinine, 170 μg/ml leupeptin, 100 μg/ml PMSF; pH 7.5). After mixing for 30 min at 4 °C, the mixture was centrifuged (10,000 × g) for 10 min, and the supernatants were collected as whole-cell extracts. The protein content was determined with Bio-Rad protein assay reagent using bovine serum albumin as a standard. The ECL Western blotting was performed as follows. An equal protein content of total cell lysates from the control, esculetin-treated sample, Taxol-treated sample, and esculetin plus Taxol-treated sample was resolved on