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Antiproliferation of HeLa cells by 3,4,5-trihydroxy-*N*-[2-*p*-tolylethyl]-benzamide is associated with induction of DNA damage and inhibition of DNA replication

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ABSTRACT

The compound 3,4,5-trihydroxy-*N*-[2-*p*-tolylethyl]-benzamide (THTEB) is one of the derivatives of tyrosol, which is *p*-tyrosol combined with gallic acid by an amide bond. In this study, THTEB displayed a significant antiproliferative effect on human cervical carcinoma (HeLa) cells. Cell cycle analysis revealed that THTEB could arrest HeLa cells in the S phase with a concomitant decrease in the cells' G_0/G_1 and G_2/M phases. According to the [^{3}H]thymidine incorporation assay results, we found that THTEB could inhibit DNA replication, which suggests that THTEB-induced S phase arrest might be the direct result of blocked DNA synthesis. However, THTEB had very weak effect on replication protein A (RPA)'s ssDNA binding activity and the topoisomerase I (topo I)-mediated DNA relaxation activity, signifying that RPA and topo I were not the main target molecules in the inhibition of DNA replication. Furthermore, by using alkaline single-cell gel electrophoresis (comet assay), we found severe DNA damage caused by THTEB. In conclusion, these results suggest that THTEB could induce tumor cell antiproliferation correlated with DNA damage and DNA replication inhibition, but the target molecule of THTEB remains elusive.

1. Introduction

To date, the cure for cancer has not yet been discovered, so research regarding anticancer drugs is becoming more and more important in the pharmaceutical industry. Almost all tumor cells have common characteristics, such as the cell proliferation-related gene has been activated, or the cell differentiation gene has been inhibited. Because of these reasons, tumor cells from mammalian species exhibit unlimited proliferation. There are many anticancer pathways, such as leading tumor cell apoptosis, the impact of the nucleic acid biosynthesis, induction of DNA structure damage, inhibition of RNA synthesis, prevention of the transcript process, or the impact of protein synthesis and function. From the perspective of cell biology, compound-induced tumor cell apoptosis (Zhang et al., 2007) and inhibition of proliferation (Jiang et al., 2005) might play important roles.

In the biological cell cycle, DNA replication requires the recruitment of multiple components during the S phase of the cell cycle, and it is a tightly regulated progress (Brush et al., 1994). In the S phase of the cell cycle, chromosomal DNA is replicated precisely once as a prelude to its segregation to the daughter cells during mitosis. If DNA replication is blocked by an inhibitor or the template is damaged by radiation or other factors, signals are generated that can induce cell-cycle arrest or apoptosis (Pizer et al., 1998).

Much of what is currently known about the mechanism of DNA replication in eukaryotic cells has come from studying simian virus 40 (SV40) and related viruses (Hurwitz et al., 1990). The *in vitro* SV40 DNA replication system has been extensively used as a model to understand eukaryotic DNA replication and for analysis of anticancer drugs (Snapka et al., 1996). In replication, replication protein A (RPA) mediates the unwinding of SV40 origin-containing DNA in the presence of SV40 T-Ag and topoisomerase. It interacts with T-Ag and the DNA polymerase α-primase complex (pol α-primase), which is necessary for the initiation of SV40 DNA replication (Collins and Kelly, 1991; Melendy and Stillman, 1993). Once replication is initiated, the elongation phase of replication is carried out by DNA pol α and δ. Three widely used S phase-specific anticancer agents, 1-β-D-arabinofuransylcytosine (ara-C), camptothecin (CPT), and doxorubicin (DOX), have been found to target three different enzymes (DNA pol α and topoisomerase I and II, respectively) (Waleed et al., 2004).

Phenolic compounds are the secondary metabolism products that have one or more substitutable hydroxyl groups in their ring structures, and are considered the most representative antioxidant agents. Phenolic compounds are widely distributed in nature; the phenolic substances are potent inhibitors of reactive oxygen

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species (ROS), e.g., salicylic acid and 2-deoxyguanosine. Currently, there is growing evidence that reactive oxygen species are involved in the aetiology of fat-related neoplasms, such as cancer of the breast and colorectum (Owen et al., 2000).*p*-Tyrosol (4-hydroxyphenylethanol) is a well-known phenolic compound that is present in different dietary sources like virgin olive oil, wine and *Rhodiola* species. It is one of the major bioactive components in *Rhodiola* species (Cui et al., 2003) and has been shown to have mild antioxidant properties with *in vitro* and *in vivo* studies (Casas et al., 2001).

Gallic acid (3,4,5-trihydroxybenzoic acid), which is naturally abundant in plants, is another well-known phenolic compound. It is known that gallic acid can prevent oxidative damage induced by ROS and cause apoptosis in some tumor cell lines (HL-60RG, HeLa, KB cells et al.) with higher sensitivity than normal cells (Inoue et al., 2002; Inoue et al., 1995). Furthermore, in recent years, several structure–activity relationship studies have demonstrated that methylation of the phenolic hydroxyl group and esterification of the carboxyl group can reduce cytotoxic activity and shows selectivity for cancer cells (Fiuza et al., 2004; Gomes et al., 2003).

So, in an effort to explore and develop an anticancer drug, we examined the activities of a series of derivatives of tyrosol and gallic acid. In our previous research, we investigated *p*-tyrosyl gallate and other derivatives, which combined *p*-tyrosol with gallic acid by esterification. *p*-tyrosyl gallate can inhibit three important functional replication proteins (topoisomerase I, RPA and pol α-primase) and has an especially strong effect on pol α-primase (Ahn et al., 2008). In this study, we examined the bioactivity of THTEB, which combined *p*-tyrosol with gallic acid by an amide bond (Fig. 1).

2. Materials and methods

2.1. Materials

PI/RNase staining buffer for cell cycle analysis was purchased from BD Biosciences Pharmingen, USA. Dimethyl sulfoxide (DMSO), ethidium bromide (EtBr) and phosphate buffered saline (PBS) (pH 7.4) were purchased from Sigma Chemical Co., USA. Eagle's minimum essential medium (EMEM), fetal bovine serum (FBS), penicillin–streptomycin, and trypsin–EDTA were obtained from Hyclone Laboratories (Logan, UT, USA). The Cell Counting Kit-8 (CCK-8) was purchased from Dojin Laboratories (Osaka, Japan). pBR322 DNA was obtained from Takara Shuzo Co., Japan, and agarose was purchased from Promega Co., USA. Topoisomerase I (topo I), human DNA polymerase α-primase (pol α-primase) and replication protein A (RPA) were prepared as described previously (Lee et al., 1991). All other chemicals were of analytical reagent grade.

Human cervical carcinoma cells obtained from the American Type Culture Collection (ATCC) were cultured in MEM medium supplemented with 10% FBS at 37 °C (5.0% CO_2) in a humidified atmosphere.

2.2. Preparation of THTEB

THTEB is *p*-tyrosol combined with gallic acid by an amide bond. The synthesis route of THTEB includes the following two steps (Fig. 1): First, 3,4,5-trimethoxybenzoyl chloride (0.01 mol), 4-methoxyphenethyl amine (0.011 mol) and potassium carbonate were stirred in H_2O/EtOAc (1:6). The reaction mixture was kept at room temperature over 24 h. The resulting mixture was filtered and the product was purified by recrystallization from EtOAc. Second, THTEB (0.003 mol) was dissolved in freshly distilled CH_2Cl_2 (20 ml). Under cooling, 1 M BBr_3 (0.015 mol) was added slowly to the solution. The mixture was stirred for 24 h at room temperature. After H_2O was added, the mixture was stirred for a few minutes. The resulting mixture was filtered and the product was purified by recrystallization from dichloromethane. A stock solution of THTEB was prepared in DMSO and kept at 4 °C. Further dilutions were made immediately prior to each experiment.

2.3. Cell viability and proliferation assay

Cell viability was determined by CCK-8 to count living cells by combining water-soluble tetrazolium salt (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium, WST-8) and 1-methoxy PMS. HeLa cells were plated at 5×10^3 cells into each well of a 96-well microplate. After the cells were incubated for 24 h, THTEB at various concentrations (12.5, 25, 50, 100, 200, and 400 μM) was added to each well as a treatment, and an appropriate volume of drug vehicle (DMSO) was used as the control. The plate was incubated for an additional 48 h. The cell viability was determined by CCK-8 from Dojindo Laboratories.

For the cell proliferation assay, cells seeded at 5×10^3 per ml media in 96-well plates were treated with (100, 200 and 400 μM) or without THTEB (control) for various times, and then 10 μl of CCK-8 reagent was added and the plates were incubated for another 2 h. WST-8 was reduced by dehydrogenases in the cells to give a yellow-colored product (formazan), which is soluble in the cell culture medium. The optical density for living cells was read at 450 nm in a multi-microplate reader (Synergy HT, BIO-TEK®) (Tominaga et al., 1999). Each experiment was repeated at least three times.

2.4. DNA content analysis

The cells (5×10^5 cells per 60 mm^2 dish) treated with or without THTEB were collected by trypsinization and washed with precooled PBS via centrifugation. The cells were suspended in PBS and fixed with 70% ethanol (v/v). Samples were washed with PBS and

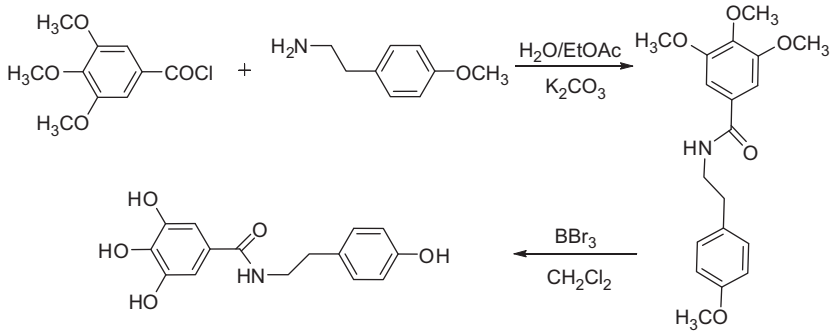

Fig. 1. Synthesis route for 3,4,5-trihydroxy-*N*-[2-*p*-tolylethyl]-benzamide (THTEB).