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B-9-3, a novel β-carboline derivative exhibits anti-cancer activity via induction of apoptosis and inhibition of cell migration in vitro

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ABSTRACT

Peganum harmala L is an important medicinal plant that has been used from ancient time due to its alkaloids rich of ß-carbolines. Harmane is a naturally occurring ß-carboline extracted from *Peganum harmala L*, that exhibits a wide range of biological, psychopharmacological, and toxicological actions. The synthesis of novel derivatives with high anti-cancer activity and less side effects is necessary. In the present study, B-9-3—a semi-synthetic compound that is formed of two harmane molecules bound by a butyl group—showed a strong anti-cancer activity against a human lung cancer cell line, a human breast cancer cell line, and a human colorectal carcinoma cell line. B-9-3 anti-proliferative effect followed a similar pattern in the three cell lines. This pattern includes a dose-dependent induction of apoptosis, or necroptosis as confirmed by Hoechst staining, flow cytometry and western blot analyses, and the inhibition of cancer cells migration that was shown to be dependent on the drug's concentration as well. Moreover, B-9-3 inhibited tube formation in human umbilical vascular endothelial cell line (HUVEC), which indicates an anti-angiogenesis activity in vitro. In summary, B-9-3, a semi-synthetic derivative of ß-carboline, has an anti-proliferative effect against tumor cells via induction of apoptosis and inhibition of cell migration.

1. Introduction

Cancer resistance to therapy is becoming a common phenomenon that threatens the current strategies adopted against this disease. As this phenomenon continues to emerge, novel molecules are needed in order to fight the evasion of tumors.

Naturally occurring phytochemicals from various plants are always an important source in the discovery of new therapeutic agents. *Peganum harmala L* is a plant that has been traditionally used as an emmenagogue and an abortifacient agent for many years (Monsef et al., 2004). Previous studies have shown that the alkaloids contained in these seeds are mostly β-carbolines: harmane, harmine, harmaline and harmalol (Kartal et al., 2003). In addition to their occurrence in plants, β-carbolines are also endogenously synthesized in mammals from tryptophan and tryptophan-derived indoleamines (Susilo and Rommelspacher, 1987). The pharmacological effects of this class of compounds are varied and include: neuro-pharmacological activities by acting as hallucinogenic and anticonvulsant agents, and possess anti-tumor, anti-viral, anti-bacterial and anti-parasitic activities (Alomar et al., 2013; Cao et al., 2007).

Moreover, there is strong evidence that these molecules are neurotoxic and may be involved in the pathogenesis of Parkinson's disease (Polanski et al., 2011). A previous study showed that the plasma levels of norharmane and harmane in Parkinsonian patients were significantly higher compared to the control group (Kuhn et al., 1995). Due to the variety of the pharmacological activities of β-carbolines family, we aspired to synthesize novel derivatives with potent anti-tumor activity and low side effects.

Chemically, β-carbolines are a large group of indole alkaloids that possess a common tricyclic pyrido [3,4-β] indole ring structure (Polanski et al., 2011). The literature reported that the protection of the nitrogen atom from the indole cycle with a methyl group renders the molecule neurostimulatant, neuroprotective and neuroregenerative of dopaminergic neurons with anti-inflammatory effects while the introduction of the same group on the nitrogen atom from the pyridine cycle results in neurotoxic potency (Polanski et al., 2010; Polanski et al., 2011). In the present study we report a newly synthesized β-carboline derivative B-9-3—which is a semi-synthetic compound that contains two molecules of harmane bound to each other by a butyl group. On one hand, the two molecules of harmane are linked at the nitrogen atoms of their indole groups as to avoid the neurotoxic effect reported on harmane (Polanski et al., 2011). On the other hand, the most promising anti-cancer leads deriving from β-carbolines were obtained through substitutions on the indolic ring (Cao et al., 2007; Ikeda et al., 2011; Ikeda et al., 2012); therefore, we believe that this cycle is the key group responsible for the anti-cancer activity of β-carbolines which may be the key group responsible for the anti-cancer activity of β-carbolines. With this knowledge, we

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synthesized B-9-3—hoping to increase the anti-cancer potency and reduce the side effects of harmane.

In this study, we investigated the anti-tumor potential of B-9-3 in three different cancer cell lines: human non-small-cell lung cancer cell line NCI-H460, human estrogen positive breast cancer cell line T47D and human colon cancer cell line HCT-116. The probable mechanisms involved in the anti-cancer effect of our compound were discussed in depth during this investigation.

2. Materials and methods

2.1. Drugs

2.1.1. B-9-3

B-9-3 was provided by "Xinjiang Huashidan Pharmaceutical Research CO., LTD" (Urumqi, CHINA). For in vitro studies, a stock solution of B-9-3 at 1 mM was made in Dimethyl sulfoxide (DMSO). Serial dilutions were made from the stock solution in serum-free media just on the day of treatment.

2.1.2. Taxol (paclitaxel)

Paclitaxel (Taxol) was purchased from HaiKou Pharmaceutical. Taxol was diluted in serum-free culture media and was given to cells at a final concentration of 300 nM.

2.2. Cell lines and cell culture

Human non-small-cell lung cancer (NSCLC) cell line NCI-H460, human breast cancer cell line T47D, and human colorectal carcinoma HCT-116 were purchased from American Type Culture Collection ATCC (Manassas, Virginia, USA). Cells were maintained in RPMI-1640 medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 2 mM glutamine, 10 mM HEPES buffer (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), 100 U/ml streptomycin and 100 U/ml penicillin at 37 °C in a humid atmosphere (5% CO_2, 95% air). For subculture, cells were detached with 0.25% trypsin-EDTA (Ethylenediaminetetra-acetic acid) solution, and 1×10^6 cells were seeded into new flasks.

2.3. MTT cell viability assay and determination of the IC50 of B-9-3

Cell proliferation was determined by the 3 [4,5 dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT, Shanghai Yijiebio). The assay was conducted according to the manufacturer's protocol. Briefly, cells were incubated with 0.5 mg/mL of MTT labeling reagent for 4 h, then MTT was removed and the formazan crystals were dissolved in DMSO for 10 min. The absorbance was read at 590 nm using a microplate reader (BioRad, USA). This experiment was performed three times.

2.4. Wound healing assay

NCI-H460, T47D and HCT-116 cells were seeded into a 24-well plate and allowed to reach 90% of confluence in a complete medium. A single scratch wound was created on confluent monolayers by using a micropipette tip (1 mm) that touched the plate as previously described (Supino et al., 2008). Then, wounded monolayers were washed with phosphate buffer saline (PBS) to remove cell debris, supplemented with serum-free medium and treated with various concentrations of the drug, and finally incubated for 24 h. Cells migrated into the wound surface and the average distance of migrating cells was determined under an IX71 Olympus inverted microscope at designated time points. Pictures from three different regions for each wound were taken. The experiment was performed three times.

2.5. Transwell migration assay

Transwell migration assay was performed as previously described (Supino et al., 2008). In brief, cells were seeded in 24-well plates in a complete medium and, after 24 h, the medium was replaced with free-serum medium and cells were treated with different concentrations of the drug for 24 h. Cells were then harvested and transferred in the upper chamber of 24-well Transwell plates (Millipore, USA) in a serum-free medium (1.2×10^5 cells/well), while the corresponding conditioned media were added to the lower chamber. Cells were treated with the drug and maintained at 37 °C for 5 h. Then, non-adherent cells were washed away by PBS, and only adherent cells were fixed in ethanol. After staining with 0.1% crystal violet, pictures of migrated cells, from 5 different areas from each well, were taken under an inverted microscope. All experiments were performed three times.

2.6. Hoechst staining

Tumor cells were seeded in 24-well plates. When they reached 70% of confluence, cells were treated with various drugs for 24 h. Then, cell apoptosis was assessed using Hoechst 33258 kit (Beyotime, Nanjing, CHINA) following the manufacturer's protocol. Briefly, the medium was removed and cells were fixed at 4 °C overnight. Afterward, cells were washed twice with PBS and stained with Hoechst 33258 for 5 min. Additionally, cells were washed twice with PBS, and each well was covered with a drop of an anti-fade mounting medium. Nuclear morphology was visually evaluated by fluorescence microscopy. All experiments were performed three times.

2.7. Annexin V-FITC/PI double staining for apoptosis detection

Tumor cells were incubated in 6-well plates with different drugs for 24 h. Cells were stained with FITC-conjugated Annexin V and propidium iodide (PI), using Annexin V-FITC Apoptosis Detection kit according to manufacturer's recommendation (UBIO, China). The percentage of apoptotic cells was determined on a flow cytometer (BD FACSCalibur; Becton, NJ, USA) using CELLQuest software (Becton Dickinson), at Nanjing Medical University-flow cytometry facility. The percentage of specific apoptosis was calculated by subtracting the percentage of spontaneous apoptosis of the relevant controls from the total percentage of apoptosis (Kong et al., 2010).

2.8. Western blot analysis

Tumor cells were incubated with different drugs for 24 h. Cell lysates were prepared in a RIPA lysis buffer (Beyotime, Nanjing, CHINA) with 1 nM of phenylmethylsulfonyl fluoride (PMSF). The protein concentration of each sample was determined with a bicinchoninic acid (BCA) assay Protein Assay Kit (Beyotime, Nanjing, CHINA). Samples containing 50 μg proteins were mixed with lamelli loading buffer at 1:3 ratio (v/v) and boiled for 5 min at 100 °C before being loaded onto a Mini PROTEAN® 3 (Bio-Rad) electrophoresis unit with a 12% SDS-polyacrylamide gel and ran at 80 V for 30 min and at 120 V for 1 h. The proteins were then transferred onto a nitrocellulose paper with a Mini Trans-Blot® Cell (Bio-Rad) at 10v for 45 min. Then, membranes were incubated in blocking solution (2% of bovine serum albumin BSA in Tris Buffered Saline-tween 20, TBST) for 2 h at room temperature with shaking, rinsed with TBST and were incubated with monoclonal antibodies to ß-actin, Bax, Bcl_2 and Caspase-3 (Cell Signaling Technology, Beverly, MA, USA) at 4 °C overnight. After rinsing three times with TBST, each time for 10 min, the membranes were incubated with secondary antibodies (horseradish peroxidase linked; Cell Signaling Technology; for 90 min at room temperature, washed with TBST three times, each time for 10 min, and reacted