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Cytotoxic activity of hirsutanone, a diarylheptanoid isolated from *Alnus glutinosa* leaves

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ABSTRACT

Background: The low efficacy of cancer therapy for the treatment of patients with advanced disease makes the development of new anticancer agents necessary. Because natural products are a significant source of anticancer drugs, it is important to explore cytotoxic activity of novel compounds from natural origin.
Purpose: The aim of this work is to evaluate the cytotoxic capacity of hirsutanone, a diarylheptanoid isolated from *Alnus glutinosa* leaves. Hirsutanone cytotoxic way of action was also studied.
Material and methods: The cytotoxic ability of *Alnus glutinosa* leaves ethyl acetate extract was studied over HeLa and PC-3 cell lines, with the MTT colorimetric assay. Hirsutanone was isolated from this extract using chromatographic methods, and its structure elucidated by spectroscopic analysis. HT-29 cell viability after hirsutanone treatment was determined using SRB assay. In order to understand hirsutanone way of action, cytotoxicity was evaluated adding the diarylheptanoid and antioxidants. DNA topoisomerase II (topo II) poison activity, was also evaluated using purified topo II and a supercoiled form of DNA that bears specific topo II recognition and binding region; topo II poisons stabilize normally transient DNA-topo II cleavage complexes, and lead an increased yield of linear form as a consequence of a lack of double-strand breaks rejoining.
Results: The diarylheptanoid hirsutanone was isolated from *Alnus glutinosa* (L.) Gaertn. (Betulaceae) leaves extract that showed cytotoxic activity against PC-3 and HeLa cell lines. Hirsutanone showed cytotoxic activity against HT-29 human colon carcinoma cells. Pre-treatment with the antioxidants NAC (N-acetylcysteine) and MnTMPyP (Mn(III)tetrakis-(1-methyl-4-pyridyl)porthyrin) reduced this activity, suggesting that reactive oxygen species (ROS) participate in hirsutanone-induced cancer cell death. Using human topo II and a DNA supercoiled form, hirsutanone was found to stabilize topo II-DNA cleavage complexes, acting as a topo II poison.
Conclusion: Our data suggest that, like curcumin, an induction of oxidative stress and topo II-mediated DNA damage may play a role in hirsutanone-induced cancer cell death. Since both compounds share similar structure and cytotoxic profile, and curcumin is in clinical trials for the treatment of cancer, our results warrant further studies to evaluate the anticancer potential of hirsutanone.

Introduction

Cancer kills more than seven million people worldwide every year (Jemal et al., 2011). The mortality rate of this disease has not changed much in the past few decades even in developed countries such as the United States (Siegel et al., 2013). Although surgery or radiotherapy as cancer therapy is effective when the disease is detected early, many cancers are still diagnosed when cells from a primary tumor have already metastasized to other parts of the body. The main form of treatment at this point is chemotherapy, which consists of delivering drugs systemically so they can reach and kill tumor cells.

Despite recent interest by pharmaceutical companies in molecular modeling, combinatorial chemistry and other synthetic chemistry techniques, natural products and medicinal plants have been found to be an important source of new drugs. Natural products are not only used as therapeutic agents, they also constitute a source of lead compounds that have provided the basis for new drugs semisynthesis or total synthesis (Newman and Cragg, 2012; Cragg et al., 2009). The role of natural products in drug discovery

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is particularly important in oncology (Newman and Cragg, 2012). There are many mechanism of action of these drugs, as inhibition of microtubule, inhibition of DNA topoisomerase II or DNA topoisomerase I inhibition (Cragg and Newman, 2005). Recent evidence suggests that the formation of reactive oxygen species (ROS) may also contribute to the cytotoxic effects of these drugs (Alexandre et al., 2006, 2007; Gorman et al., 1997). The induction of oxidative stress by pro-oxidant agents is indeed emerging as an attractive cytotoxic strategy (Pelicano et al., 2004; Renschler, 2004; Schumacker, 2006; Lopez-Lazaro, 2007, 2010).

Alnus glutinosa (L.) Gaertn (alder), is a common Betulaceae widely distributed in Europe. Alder bark and leaves are traditionally used in folk medicine (Grieve, 1984). A decoction of leaves had been used for treating several types of cancer (Hartwell, 1967–1971). Some of its constituents exhibit various biological properties, including anti-inflammatory and cytotoxic activities (Acero and Muñoz-Mingarro, 2012). *Alnus* genera phytoscreening had led the identification of numerous diarylheptanoids. Diarylheptanoids, such as curcumin, belong to a phenolic class of natural products based on 1,7-diphenylheptane skeleton. In the last decade, numerous reports has been published on the cytotoxic activity of curcumin, and several clinical trials are currently ongoing or recruiting participants to evaluate the anticancer activity of this natural product (Lopez-Lazaro, 2008; Hatcher et al., 2008). Curcumin has been shown to interfere with multiple signal pathways, including apoptosis, proliferation cell cycle, cell survival, inflammation, invasion and metastasis (Bachmeier et al., 2010). One of the main molecular mechanisms involved in curcumin apoptotic and antimetastasic effect is the inhibition of nuclear factor kappa B (NF-kappaB) transcription. Metastasis related proinflammatory cytokines CXCL-1 and -2 are also modulated as they are NF-kappa targets (Bachmeier et al., 2009). Evidence also suggests that the generation of ROS and the induction of topoisomerase II-DNA complexes may play a role in curcumin-induced cancer cell death (Kuo et al., 1996; Martin-Cordero et al., 2003; Lopez-Lazaro et al., 2007).

In this work, we have isolated the diarylheptanoid hirsutanone from a cytotoxic extract of *Alnus glutinosa* and shown that the formation of ROS, and the inhibition of topo II may participate in its cytotoxic activity. Because curcumin and hirsutanone share a similar structure and cytotoxic profile, our results warrant future studies to evaluate the anticancer potential of hirsutanone.

Material and methods

Cell culture and chemicals

The HT-29 human colon carcinoma cell line was obtained from the European Collection of Cell Cultures (ECACC, Ref. 91072201) and was grown in McCoy's 5a (Sigma), supplemented with 2 mM Glutamine and 10% Fetal Bovine Serum (FBS). The human cervical carcinoma HeLa cell line and the human prostate adenocarcinoma PC-3 cell lines were obtained from the American Type Culture Collection (ATCC CCL-2 and CRL-1435 respectively). HeLa cells were grown in Eagle Minimal Essential Medium (EMEM), supplemented with 10% FBS, 1% non-essential amino acids (100×), 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine. PC3 cells were cultured in Coon's modified Ham's F12, supplemented with 45 mg/l ascorbic acid, 18 mg/l inositol, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 7% FBS. All cancer cell lines were cultured at 37 °C in a humidified 5% CO_2 atmosphere.

Purified human topo II and supercoiled pRYG DNA were purchased from TopoGen, Inc. (Columbus, OH). MnTMPyP (Mn(III)tetrakis-(1-methyl-4-pyridyl)porthyrin) was obtained from Biomol International. Other chemicals, including curcumin, etoposide, 5-fluorouracil, NAC (N-acetylcysteine), and proteinase K, were obtained from Sigma.

Plant material and extract preparation

Alnus glutinosa (L.) Gaertn. (Betulaceae) was collected in July 2007 in San Agustín de Guadalix (Madrid, Spain) (40°41′ N 3°36′ W). A voucher specimen (2642/09) was deposited in the Faculty of Pharmacy Herbarium, University San Pablo, CEU Madrid. The leaves of this plant were dried at room temperature, powdered, and extracted with ethyl acetate under reflux for 2 h (Buchi, B-811). The extract was concentrated to dryness under vacuum and stored at 4 °C until use. The yield of extraction was 4.7%.

Extract fractionation and hirsutanone isolation

The ethyl acetate extract (2 g) was subjected to silica gel column chromatography and eluted with a step gradient of $CHCl_2$–MeOH (99:1, 95:5, 80:20, 70:30, 1:1) and MeOH 100% (v/v) to yield 258 fractions of 20 ml each. Fractions 170–219 (343 mg) were again subjected to silica gel column chromatography with $CHCl_2$–MeOH (95:5) to give compound 1 (20 mg). This compound was characterized by spectroscopic analyses, including ^{1}H NMR, ^{13}C NMR and EIMS, and identified as 1,7-di-(3′,4′-dihydroxyphenyl)-4-hepten-3-one (hirsutanone) (Martin-Cordero et al., 2001).

Cytotoxicity assays (MTT and SRB assay)

Cell viability was assessed using either the MTT (Twentyman and Luscombe, 1987) or the SRB assay (Vichai and Kirtikara, 2006). In MTT assay exponentially growing cells (HeLa and PC3) were seeded in 96-well plates at a density of 2.5×10^3 cells/well. After 24 h, cells were treated with the extract or reference compounds and incubated for 72 h (MTT). After treatment, cells were washed twice with PBS. Then, 50 μl/well of 3-(4,5-dimethylthiazol-2-yl)-2,5,diphenyl tetrazolium bromide (MTT) reagent (1 mg/1 ml in PBS), and 150 μl/well of prewarmed medium were added and cells were incubated for 4 h. The medium was then aspirated, and the formazan product generated from the viable cells, was dissolved in DMSO before being measured at 570 nm using an automatic plate reader (Opsys, MR). All experiments were performed at least three times. The percentage absorbance related to control was plotted against concentration. Concentration of extract required to inhibit 50% of cell growth (IC_{50}) was calculated. Data are expressed as means ± SEM.

In the SRB assay, exponentially growing HT-29 cells were seeded in 96-well plates at a density of 10×10^3 cells/well. After 24 h, cells were treated with the extract or isolated compounds and incubated for 48 h. Then cells were fixed by adding 50 μl/well of cold 50% (w/v) trichloroacetic acid (TCA) and incubated for 1 h at 4 °C. The supernatant was then discarded and plates were washed five times with deionised water and dried. 100 μl/well of SRB (sulforhodamine B) solution (0.4%, w/v in 1% acetic acid) were added and plates were incubated for 30 min at room temperature. Unbound SRB was then removed by washing five times with 1% acetic acid. The plates were then air-dried and protein-bound dye was dissolved in 10 mM Tris base solution for OD determination at 492 nm, using a microplate reader (Vichai and Kirtikara, 2006). Cell viability was expressed as percentage in relation to controls. Data were averaged from at least three independent experiments and are expressed as means ± SEM.

To assess whether the cytotoxicity of hirsutanone was mediated by a pro-oxidant mechanism, HT-29 cells were treated with 10 μM hirsutanone, 10 μM curcumin, and 10 μM 5-fluorouracil for 48 h, in the absence and presence of 5 mM NAC (N-acetyl-l-cysteine) and 5 μM MnTMPyP (Mn(III)tetrakis-(1-methyl-4-pyridyl)porthyrin). The antioxidants NAC and MnTMPyP were added 0.5 h before