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A novel parthenin analog exhibits anti-cancer activity: Activation of apoptotic signaling events through robust NO formation in human leukemia HL-60 cells

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ABSTRACT

This study describes the anti-cancer activity of P19, an analog of parthenin. P19 induced apoptosis in HL-60 cells and inhibited cell proliferation with 48 h IC50 of 3.5 μM. At 10 mg/kg dose, it doubled the median survival time of L1210 leukemic mice and at 25 mg/kg it inhibited Ehrlich ascites tumor growth by 60%. Investigation of the mechanism of P19 induced apoptosis in HL-60 cells revealed that N-acetyl-L-cysteine (NAC) and s-methylisothiourea (sMIT) could reverse several molecular events that lead to cell death by inhibiting nitric oxide (NO) formation. It selectively produced massive NO in cells while quenching the basal ROS levels with concurrent elevation of GSH. P19 disrupted mitochondrial integrity leading to cytochrome *c* release and caspase-9 activation. P19 also caused caspase-8 activation by selectively elevating the expression of DR4 and DR5. All these events lead to the activation of caspase-3 leading to PARP-1 cleavage and DNA fragmentation. However, knocking down of AIF by siRNA also suppressed the apoptosis substantially thus indicating caspase independent apoptosis, too. Further, contrary to enhanced iNOS expression, its transcription factor, NF-κB (p65) was cleaved with a simultaneous increase in cytosolic IκB-alpha. In addition, P19 potently inhibited pro-survival proteins pSTAT3 and survivin. The multimodal pro-apoptotic activity of P19 raises its potential usefulness as a promising anti-cancer therapeutic.

1. Introduction

Cancer cells accumulate several genetic and epigenetic mutations to cleverly evade self demise by deregulating apoptosis and setting a stage for initiation, promotion and progression of the malignant growth [1]. Agents that can trigger the process of apoptosis in cancer cells are therefore, considered potentially important for the development of anti-cancer chemotherapeutics [2]. In this concern, we focused our attention on parthenin, a major sesquiterpene lactone from the obnoxious weed *Parthenium hysterophorus* Linn. (Asteraecae) that grows wild in different regions of India. The plant causes contact dermatitis and allergic rhinitis in animals [3]. Despite the fact that *Parthenium* is considered a toxic plant its traditional uses are amply reported in literature [4]. One of its chemical constituent parthenin is reported to be responsible for cytotoxic effects of this plant [5,6]. Parthenin has also been reported to exhibit anti-tumor activity in mice [7]. In view of the reported toxicity of parthenin we attempted to prepare various analogs of parthenin modifying its functional groups to obtain some potent anti-cancer analog of lesser toxicity. In this endeavor we screened several semi-synthetic analogs of parthenin primarily for their apoptotic index and selected P19 among them as the prospective pro-apoptotic candidate for its detailed studies to develop it into a promising anticancer therapeutic lead. About 50% of the cancers are known to have p53 mutated. Therefore, we employed p53 null, human acute myeloid leukemia HL-60 cells [8]. Thus, the agents that can induce apoptosis via a *p53*-independent pathway should have a broader therapeutic potential not only in AML patients but also in other cancer diseases.

Several earlier studies have demonstrated that nitric oxide (NO) is an important signaling messenger that plays an important role in many physiological and pathological conditions. Endogenous NO is generated from L-arginine by three major types of NO synthases (NOS), i.e. endothelial NOS, neural NOS, and inducible NOS (iNOS) while another form of NO has recently been reported from

Abbreviations: AIF, apoptosis inducing factor; AML, acute myeloid leukemia; DR4, death receptor 4; DR5, death receptor 5; DRP-1, dynamin related protein-1; GSH, glutathione reduced; iNOS, inducible nitric oxide synthase; NAC, N-acetyl-L-cysteine; NF-κB, nuclear factor-kappa B; PARP-1, poly (ADP ribose) polymerase-1; pSTAT3, phosphorylated signal transducer and activator of transcription protein-3; ROS, reactive oxygen species; sMIT, s-methylisothiourea; TNFR1, tumor necrosis factor receptor 1.

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mitochondria [9,10]. Several studies have amply demonstrated that NO can induce apoptosis in a variety of tumor cells [11,12], by mechanisms involving induction of stress proteins, mitochondrial disruption, release of cytochrome *c*, and caspase activation [13]. In the recent past, we reported a lignan composition from *Cedrus deodara* that caused NO-mediated apoptotic cell death of Molt-4 cells by activating both intrinsic and extrinsic pathways [14]. Nitric oxide in vivo may contribute to paracrine tumor-suppressive activities by triggering various signaling pathways. A known example is tamoxifen, which potently induced iNOS and NO formation in myoepithelial cells in culture in an estrogen receptor-β-dependent manner [15]. This chemotherapeutic is used for high risk population of women with familial history of breast cancer. One potent transcription factor accounting for iNOS induction is the nuclear factor-kappa-B (NF-κB), which is constitutively activated in most of the cancer cell lines and in several cancer tissues while its level is very low in resting cells [16]. Cells that express constitutively activated NF-κB are resistant to various chemotherapeutic agents and radiation treatment [16]. Many previous studies have implicated NF-κB in the development and progression of cancer. NF-κB is also known to regulate the expression of several anti-apoptotic genes such as IAPs, Bcl-2, Bcl-xL and survivin [17]. In addition NF-κB regulates the expression of several genes involved in cancer cell proliferation, interestingly, some of these genes are also regulated by STAT3 (signal transducer and activator of transcription protein-3). In fact STAT3 and NF-κB are closely linked and both these transcription factors require each other for their persistent activation in the cancer cells [18]. STAT3 is found to be crucial for growth of several cancer types but its expression is dispensable for normal cells in postembryonic stage [19]. This makes NF-κB and STAT3 an ideal target for development of anticancer therapeutics. The present studies describe the multi-modal action of P19 in killing cancer cells by targeting several such apoptotic signaling pathways where the selective generation of NO appears indispensable initiating event in activating pro-apoptotic cascades while simultaneously inhibiting the activity of NF-κB and STAT3.

2. Materials and methods

2.1. Chemicals and antibodies

Dihydrorhoethidium (DHE), 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA), 4,5-diaminofluorescene-diacetate (DAF-2-DA), L-buthionine-S,R-sulfoximine (BSO), ethidium bromide, propidium iodide (PI), DNase-free RNase, proteinase K, 3-(4,5,-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT), N-acetyl-L-cysteine (NAC), s-methylisothiourea (sMIT), staurosporine, camptothecin, Fetal bovine serum were purchased from M/s Sigma–Aldrich, USA; other reagents used were of analytical grade and available locally. Annexin V-FITC apoptosis detection kit, Mitochondrial Membrane Sensor Kit and Apo Alert glutathione detection kit were obtained from M/s BD Biosciences while Apoalert caspases assay kits were from M/s B.D. Clontech; Anti-human antibodies to Bax (#SC-20067), Bid (#SC-6538), DRP-1 (#SC-21804), PARP-1 (#SC-8007), Bcl-2 (SC-7382), TNFR1 (#SC-1070), FAS (#SC-8009), DR4 (#SC-6824), DR5 (#SC-7192), NF-κB p65 (#SC-8008), NF-κB p50 (#SC-8414), pSTAT-3 (Ser727) (#SC-21876), CASPASE-3 (#SC-7272), CASPASE-8 (#SC-56070), CASPASE-9 (#SC-56077), AIF (SC-5586), AIF siRNA (h) (SC-29193) survivin (#SC-8806), actin (#SC-8432), goat anti-rabbit IgG-HRP (#SC2030) and goat anti-mouse IgG-HRP (#SC2031), were from M/s Santa Cruz, USA; iNOS (#610432, clone 54) and cytochrome *c* (#556433, clone 7H8.2C12) were from M/s BD, Pharmingen, USA; actin (#CP01), iNOS (#482755) and rabbit anti-goat IgG-HRP (#401504) were from Calbiochem, Germany; electrophoresis reagents and protein markers were from M/s BIO-RAD, USA while Hyper film and ECL reagents from M/s Amersham Biosciences, UK. The sources for other chemicals and reagents are same as used earlier [20,21].

2.2. Synthesis of parthenin analog P19

Parthenin, a sesquiterpene lactone was isolated from a plant weed *P. hysterophorus*. P19 [2′-(4-chlorophenyl)-3-(4-chlorophenylidene)-5,10-dimethyl-12-methylene-decahydro-1,2-(1′,3′-dioxocyclopenta[c])azuleno(4,5-b)furan-4,11-dione] (Scheme 1) was synthesized from parthenin as reported earlier [22].

2.3. Cell culture, growth conditions and treatment

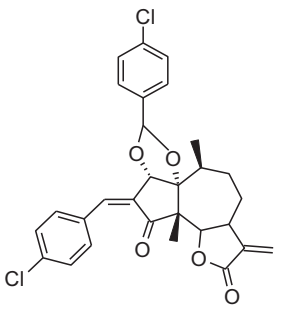
Human promyelocytic leukemia cell line HL-60 was obtained from NCI, USA; MCF-7 and HeLa cells were obtained from NCCS Pune, India; Human Gingival Fibroblast (hGF) cell line developed from healthy gingival [23] was provided kindly by Dr. Anil Balapure, Scientist, CDRI, Lucknow, India. The cells were grown in RPMI-1640 medium containing 10% FCS, 100 units pencillin/100 μg streptomycin per ml medium in CO_2 incubator at 37 °C with 95% humidity and 5% CO_2 gas environment. Cells were treated with P19 dissolved in DMSO while the untreated cultures received only the vehicle (DMSO, <0.2%, v/v).

2.4. Housing and care of animals

Swiss albino mice (22–25 g), DBA/2 female mice and CDF1 male mice (18–23 g) were housed in standard size polycarbonate cages fed with standard pellet diet (Gold Muhor, Lipton India Ltd.) and autoclaved water was given ad libitum. They were housed in controlled conditions of temperature (25 ± 2 °C), humidity (50–60%) and 12:12 h of light:dark cycle. The studies and number of animals used were approved by the institutional ethics committee.

2.5. Cell proliferation assay

Cell proliferation was determined using 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) as described earlier [20]. HL-60 cells (2.0×10^4/200 μl) and adherent cultures (10^4/200 μl) of MCF-7, HeLa and hGF cells in 96 well culture plates were treated with various concentrations of P19 for 48 h or various time periods. The MTT formazan crystals formed were dissolved in 200 μl of DMSO; OD measured at 570 nm. The cytotoxicity of P19 was expressed as the relative viability (% of untreated control cells).



Scheme 1. Structure of parthenin analog P19.