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RESEARCH ARTICLE

Strong Anticancer Potential of Nano-triterpenoid from *Phytolacca decandra* against A549 Adenocarcinoma via a Ca^{2+}-dependent Mitochondrial Apoptotic Pathway

Jayeeta Das, Sreemanti Das, Avijit Paul, Asmita Samadder, Anisur Rahman Khuda-Bukhsh*

Cytogenetics and Molecular Biology Laboratory, Department of Zoology, University of Kalyani, Kalyani 741235, India

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Abstract
We isolated a triterpenoid from an ethanolic extract of *Phytolacca decandra* and nanoencapsulated it with biodegradable nontoxic polymers of poly(lactide-*co*-glycolide) to examine if the nanoform of this hitherto unexplored betulinic-acid derivative (NdBA) could produce a stronger anticancer effect by rendering better drug bioavailability and targeted delivery than the nonencapsulated betulinic-acid derivative (dBA). The nanoparticles were characterized with the help of physicochemical and morphological studies involving dynamic light scattering and atomic force microscopy. A549 cancer cells were exposed to NdBA and dBA at the IC_{50} doses of 50 μg/mL and 100 μg/mL, respectively. Mitochondrial dysfunction-mediated apoptosis was determined by examining the changes in the intracellular calcium content, the reactive oxygen species accumulation, the cytochrome *c* release, the upregulation of Bcl-2-associated-X protein (Bax) and caspase 3, the downregulation of B cell lymphoma 2, and the mitochondrial membrane potential ($\Delta\Psi_m$) depolarization. Apoptosis was also verified by acridine orange staining observed under fluorescence microscopy and annexin V-fluorescein isothiocyanate/propidium iodide staining through flow cytometric studies. The levels of

* Corresponding author. Cytogenetics and Molecular Biology Laboratory, Department of Zoology, University of Kalyani, Kalyani 741235, India.
E-mail: prof_arkb@yahoo.co.in, khudabukhsh_48@rediffmail.com (A.R. Khuda-Bukhsh).

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intracellular adenosine triphosphate/adenosine diphosphate ratio decreased, and the ATPase activity increased more strikingly in A549 cells exposed to NdBA than in A549 cells exposed to dBA. Overall results showed that both drugs directly target the mitochondrial oxidative phosphorylation system, with NdBA having a stronger effect, indicating NdBA to be a better candidate for the development of an anticancer drug for use against lung adenocarcinomas.

1. Introduction

Cancer is one of the most dreaded diseases worldwide. Lung cancer is a common cancer type, but still the patients continue to have a poor prognosis. As cancer cells are endowed with the property of uncontrolled proliferation and growth, search for agents that can trigger apoptosis or programmed cell death in tumor cells has become a major goal in anticancer drug discovery [1]. In response to apoptotic stimuli triggered by anticancer drugs, several proteins (such as cytochrome *c*) are released from the mitochondrial intermembrane space into the cytoplasm that mediates the activation of caspase cascades, which in turn ultimately promotes apoptosis. Calcium is a key regulator of cell survival because cytosolic free calcium concentration is a major regulatory factor for large number of cellular processes such as muscle contraction, secretion, cell differentiation, and apoptosis. However, sustained elevation of intracellular calcium plays a role in cell death as well [2]. The proapoptotic effects of calcium are mediated by a diverse range of calcium-sensitive factors that are compartmentalized in various intracellular organelles, including endoplasmic reticulum (ER) and mitochondria [3]. Excessive calcium load to the mitochondria may induce apoptosis by impairing mitochondrial function, for example, generation of reactive oxygen species (ROS) by damaged respiratory chain [4]. Calcium accumulation has also been reported to stimulate mitochondrial ROS production in cells [5,6]. Mitochondrial calcium uptake also triggers significant membrane depolarization, resulting in a decrease in the mitochondrial membrane potential [3].

Many plant-derived compounds are known to have curative potentials against cancer [7—9]. The World Health Organization estimated that approximately 80% of world's inhabitants rely mostly on traditional medicines for their primary healthcare [10]. The root extract of *Phytolacca decandra*, a plant commonly known as "Chui-Xu Shang-Lu" in China, has been used for centuries as a traditional folk medicine for the treatment of tumors, edema, bronchitis, and abscesses [11]. Recently, we studied the anticancer potentials of the ethanolic root extract of *P decandra* and the nanoencapsulated form of the whole extract [8]. Yet, no work had earlier been carried out on any of the major ingredients contained in the crude ethanolic extract of PD or the nanoencapsulated form of any such individual ingredient. However, the extract of a congeneric species, *Phytolacca americana*, has been reported to contain triterpene saponins (phytolacca saponin), phytolaccagenic acid, phytolaccagenin, etc. [12]. We isolated one of the major ingredients of PD, namely, 1-isopropenyl-5*a*,5*b*,8,8,11*a*-pentamethyl-1,2,3,4,5,5*a*,6,7,7*a*,8,11,11*a*,11*b*,12,13,13*a*,13*b*-octadecahydro cyclopenta[*a*]chrysene-3*a*-carboxylic acid, which is presumably a derivative of betulinic acid (dBA) and which had not been tested for its anticancer potential. We therefore became interested to study the possible anticancer efficacy of dBA and also wanted to ascertain if the poly(lactide-*co*-glycolide) (PLGA) nanoencapsulation of dBA (NdBA) could accentuate its anticancer potential.

Therefore, the aims of the present study were to determine: (1) if both dBA and NdBA induced apoptosis in human lung adenocarcinoma (A549) cells *in vitro*, which often defy any such chemotherapeutic venture; (2) if the active ingredient and its nanoencapsulated form showed any apoptotic ability, and whether NdBA had a stronger effect than dBA; (3) if the apoptotic phenomenon was associated with change in calcium content; and finally, (4) if both dBA and NdBA modulated the intracellular calcium content, to ascertain the possible role of Ca^{2+} ion in the mitochondrial apoptotic pathway.

2. Materials and methods

2.1. Reagents

All chemicals and reagents used in this study were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Cell culture

Human nonsmall cell lung carcinoma A549 cells were procured from the National Center for Cell Science (Pune, India). The cells were cultured at 5×10^5 cells/mL in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 1% PSN antibiotic at 37 °C, 5% CO_2 for our experimental purpose.

2.3. Source of *P decandra* and identification of its major bioactive component

The ethanolic root extract of *P decandra* used as homeopathic mother tincture was manufactured and supplied by Boiron Laboratories (Lyon, France). The ethanolic extract was evaporated at 60 °C (to remove alcohol from the extract). The yield of the extract was about 52%; it was isolated using the standard column chromatography method over silica gel using a mixture of chloroform and hexane (9:1) and was sent for mass spectroscopy, Fourier transform infrared, and nuclear magnetic resonance analyses [8].