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Photochemotherapy using natural anthraquinones: Rubiadin and Soranjidiol sensitize human cancer cell to die by apoptosis

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Summary Over the past decade the science has studied synthetic photosensitizers used in photodynamic therapy (PDT) or photochemotherapy as anticancer candidates. In this context, compounds extracted from vegetable species present interesting potential in the cancer field. In our laboratory, we studied *Heterophyllaea pustulata* a phototoxic shrub that habit the northwest of Argentina. From this vegetal, by *in vitro* germination, we obtained Rubiadin and Soranjidiol, two anthraquinones that exhibited significant photocytotoxicity on human cancer cells. In addition, the fraction obtained from callus cultures allowed us to get a satisfactory content of these compounds compared to those found from the original plant. Under PDT regimen, we found that cell destruction resulted in a dose-dependent manner and occasioned apoptosis on photosensitized cells. Biochemical analysis revealed the involvement of caspase-3, PARP cleavage and DNA fragmentation in Rubiadin induced apoptosis. Moreover, Soranjidiol-PDT led to μ-calpain-induced apoptosis involving caspases-3-independent DNA fragmentation. We also showed that both anthraquinones are cytoplasmatically distributed and out of nucleus. In addition, we demonstrated a synergic cytotoxic effect when we combined them.

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Our data demonstrated that Rubiadin and Soranjidiol could be further considered as natural photocytotoxic compounds against cancer cells and callus cultures are a plausible source of these anthraquinonic compounds.

Introduction

Photodynamic therapy (PDT) or photochemotherapy is a clinically approved, minimally invasive therapeutic procedure that can exert a selective cytotoxic activity toward malignant cells. The procedure involves administration of a photosensitizing agent (PS) followed by irradiation at a wavelength corresponding to an absorbance band of the sensitizer. This creates the photodynamic reaction which is tumor and vascular ablative [1,2].

Diverse natural PSs as hypericins, hypocrellins and pheiochrome showed great activity under PDT protocols [3]. Other derivatives such as paclitaxel and aloe-emodin occasioned interesting photodynamic effect on tumor cell cultures [4,5].

Due to the great demand of vegetal compounds that has the international trade; the plant cell cultures represent new technologies that extend and enhance the usefulness of plants as sources of natural valuable compounds [6].

One type of *in vitro* cultures is the calli, characterized for being a disorganized mass of undifferentiated tissue with active dividing cells [7]. This biotechnological tool has been mainly used for the synthesis and extraction of bioactive natural products, such as therapeutic drugs.

Heterophyllaea pustulata Hook f. (Rubiaceae) is a phototoxic plant that grows in the Andean northwest of Argentina. The ingestion of different parts of the plant produces toxic effect such as dermatitis and blindness on bovine, goat and equine cattle [8,9]. We have reported previously that anthraquinones (AQs) isolated from leaves and stem of *H. pustulata* exhibited photosensitizing properties by generation of singlet oxygen and/or superoxide anion radical [10]. In other study, we observed that these AQs present increased photodynamic activity on cancerous cells [11]. In addition, we showed that AQs occasioned photosensitization on Balb/c mice [12]. In PDT, the current studies are centralized in the characterization of new synthetic and/or natural drugs that once photoactivated induce apoptosis, necrosis or autophagy [13,14].

Apoptotic cell death is morphologically characterized by chromatin condensation, cleavage of chromosomal DNA into internucleosomal fragments, cell shrinkage, membrane blebbing, formation of apoptotic bodies without plasma membrane breakdown. At the biochemical level, apoptosis entails the participation of caspases, a family of cysteine dependent aspartate-specific proteases, which act in two main converging pathways, extrinsic and intrinsic and this signaling finish with the activation of executioner caspases, caspase-3 and -7 that cleavage and inactive defined substrates implicated in cells protection as (ADP-ribose) polymerase (PARP). In contrast, apoptosis may occur by caspase-independent pathway implicating apoptosis inducing factor (AIF) and endonuclease G (Endo G) [15]. In addition to caspases, other proteases are implicated in the cellular death, such as calpains. These proteases belong to a family of calcium activated cystein proteases and have function in a wide variety of biological processes, including cell division, differentiation, migration, and apoptosis [16].

While the majority of approved PDT protocols treat superficial lesions of skin and luminal organs, interstitial and intra-operative approaches have been investigated for the ablation of a broad range of superficial or bulky solid tumors such as breast cancer [17,18]. Breast cancer is the most common women's cancer in the world. In recent years possibilities of PDT using in breast cancer treatment are analyzed [26]. In this investigation, our studies were mainly focused on the efficacy's assessing of natural photosensitizers, Rubiadin and Soranjidiol obtained from *H. pustulata* callus cultures, on human breast cancer cell lines. The viability, AQs intracellular localization and phototoxicity, exploring the cell death mechanism induced on cancer cells by the AQ-PDT treatment are analyzed. Also an alternative PDT combination, on which the synergic, antagonic or additive Rubiadin- and Soranjidiol-effect was examined.

Materials and methods

Recollection and *in vitro* germination of *H. pustulata* seeds

The seeds of *H. pustulata* were collected in Salta province, Argentina, in June. The material was identified by Prof. Dr. Gloria Barboza Instituto Multidisciplinario de Biología Vegetal, Consejo Nacional de Investigaciones Científicas y Técnica, Universidad, Nacional de Córdoba (IMBIV, CONICET, UNC). The seeds were surface disinfected by immersion in 70% ethanol for 1 min followed by treatment with 30% sodium hypochlorite (NaClO) for 5 min. To remove the traces of chlorines, they were washed twice with sterile distilled water. Then, seeds were transferred to Murashige and Shoog (MS) medium [19] supplemented with vitamins solution pH 5.7—5.8 and they were incubated at 28 °C in a culture chamber under a 16 h photoperiod.

Callus induction from different explants of *H. pustulata*

Leaf, root and stem explants derived from plants of three month age were excised longitudinally and placed on basal MS medium supplemented with 1 mg/L of 2,4-dichlorophenoxyacetic acid (2,4-D, Sigma) for inducing callus. They were incubated in darkness in growth chamber at 28 °C. To maintain the callus culture, the proliferated calli were regularly subcultured every 3 week intervals on fresh medium with the same composition supplemented with

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