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In vitro studies of antifibrotic and cytoprotective effects elicited by proto-berberine alkaloids in human dermal fibroblasts

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ABSTRACT

Background: The pathogenic mechanisms of skin fibrosis are still not completely understood, unlike the profibrotic role played by inflammatory cytokines and transforming growth factor-β1 (TGF-β1). Few antifibrotic drugs are available. Nevertheless, folk medicine suggests numerous treatments of fibrotic conditions. Based on information from folk medicine and literature, the hypothesis was made that proto-berberine alkaloids could act as antifibrotic and cytoprotective agents.
Methods: The effects of berberine, dihydroberberine, canadine, stylopine, and coptisine were investigated on an *in vitro* model of fibrosis purposely set up. The study is based on the use of human dermal fibroblasts (HDF). The ability of the proto-berberine alkaloids investigated to modulate mitochondrial dehydrogenase activity, cell proliferation, collagen production, and inflammatory cytokine (IL-1β and IL-6) production was tested on HDF cells grown under standard growth conditions, in the presence of 100 μM H_2O_2, simulating oxidative stress conditions, and in the presence of 34 ng/ml TGF-β1, simulating fibrotic conditions. Antiradical activity was assayed as well, as it could contribute to cytoprotection.
Results: Each alkaloid tested showed peculiar effects on HDF. In particular, all of the alkaloids tested, with the exception of coptisine, inhibited TGF-β1-induced collagen production.
Conclusions: Due to its irritant effects and the lack of desired properties, coptisine has low exploitation potentialities. The other proto-berberine alkaloids investigated resulted all endowed with activities for which they can be exploited as antifibrotic and cytoprotective agents. Stylopine globally proved to be the most promising compound, being endowed with revitalizing, anti-inflammatory, antifibrotic and wound-healing promoting activities, and showing no toxic effects.

Introduction

Fibrosis is characterized by the accumulation of extracellular matrix (ECM) components and the impairment of normal tissue architecture. Alteration of the balance between production and degradation of connective tissue components determines the pathogenesis of the fibrotic process, thus resulting in an increase of cell numbers which constitute connective tissue (fibroblasts, connective cells, adipocytes) and ECM components (collagen, glycoproteins, proteoglycans) [1]. Hypertrophic scars, keloids and systemic scleroderma are examples of skin fibroses.

Skin, like other tissues, reacts to a wound by scarring. Scar formation, which often ends the wound healing process, is frequently accompanied by functional and esthetic problems. Functional impairments may arise when the scarring process involves, for example, fingers and fingertips or are close to a joint, in such cases the contracture of tissue may limit movements. When scars affect facial skin, in addition to functional problems (i.e. breathing or eating may result to be compromised in some way) esthetic issues enhance the need of properly solving the problem. Traumas and burn injuries affecting the deep dermis often result in the formation of hypertrophic scars, which are characterized by being raised, reddened, itchy and painful [2,3]. Hypertrophic scars and keloids represent two types of excessive scarring formation and cutaneous fibrosis. They appear to be morphologically similar, even if not clinically. However keloids, unlike hypertrophic scars, progress beyond the boundaries of the original injury [4].

Systemic scleroderma is characterized by a thickening and hardening of the skin. It may not be limited to skin, but depending

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on its severity, visceral organs may be affected to a different extent. Endothelial cells, which constitute the internal lining of blood vessels, are the major target of autoimmune aggression. Consequently, microcirculation is impaired, making it difficult for blood to reach the skin and/or other organs, thus working as a stimulus to increase collagen production. This in turn leads to fibrosis of the organs affected [5].

Although nowadays pathogenic mechanisms of fibrosis are not completely known, several studies agree on the importance of the profibrotic role played by cytokines, such as interleukins 6 (IL-6) and 1β (IL-1β), and transforming growth factor-β1 (TGF-β1) [6,7]. In particular, TGF-β1 is known to stimulate collagen production (especially type I collagen) which is responsible for the disruption of the normal architecture in a fibrotic tissue [8].

Further studies highlight that several processes involved in scarring are controlled by reactive oxygen species (ROS), which are released by macrophages when they have migrated to the injured site. Excessive concentration of ROS species leads to oxidative stress, which in turn impairs and slows down the wound healing process [9,10].

Other cellular and biochemical processes occur during skin regeneration and repair events, both in physiological conditions and in the presence of lesions. Tissue repair processes following a lesion to the skin include the modulation of cell proliferation, migration and viability. Mitochondrial dehydrogenase activity can be taken as a first indicator of cell viability. It is known that changes in the aforementioned processes are involved in numerous skin regeneration and repair dysfunctions, such as skin aging, skin inflammation, skin irritation, skin fibrosis and other fibrotic conditions [1,11].

On the one hand, few antifibrotic drugs are available at present, although fibroses have a strong clinical and social impact. As in other pathologies that are difficult to manage, the treatment of fibrotic diseases may take advantage of the beneficial effects of medicinal plants, and compounds isolated therefrom, as suggested by folk medicine. Among natural alkaloids, berberine is known to exert cytoprotective and antifibrotic effects on several organs, such as the liver [12–14], heart [15,16], kidney [17,18], and lung [19]. To the best of our knowledge, no data is reported on berberine effects on skin fibrosis or human dermal fibroblast (HDF) cells, as well as no data regarding other proto-berberine alkaloids.

Based on information coming from folk medicine and suggestions taken from literature, the hypothesis was made that proto-berberine alkaloids could act as antifibrotic and cytoprotective agents. Such a hypothesis supplied the motivation for the present study, as the molecules under investigation are potentially suitable to aid skin regeneration, healing and remodeling processes.

The present work was therefore aimed at investigating the effects of some proto-berberine alkaloids (berberine, dihydroberberine, canadine, stylopine and coptisine) on an *in vitro* model of human skin fibrosis set up to study the problem at hand. The above alkaloids naturally occur in a wide number of plant species. Among them, several plants belonging to the *Papaveraceae* family are well-known in folk medicine for the treatment of skin lesions. Fig. 1 illustrates the chemical structures of the five proto-berberine alkaloids investigated in the present study.

Materials and methods

In order to assess the antifibrotic and cytoprotective activities of the substances under investigation, we set up an *in vitro* model consisting of a battery of assays based on HDF, which exploits experimental systems well accepted in literature as suitable models of fibrosis [7].

In particular, the primary aim of the study was to assess whether and how much the proto-berberine alkaloids investigated were able to modulate (1) mitochondrial dehydrogenase activity (optimal indicator of cell viability), (2) cell proliferation (obtained through cell counting), (3) collagen production, and (4) inflammatory cytokine production (among which IL-1β and IL-6 appear to be the most relevant ones). These experiments were carried out on HDF cells under different growth conditions: (a) in standard growth conditions (see the "Cell cultures and treatments" section below), (b) in the presence of 100 μM H_2O_2, to evaluate cytoprotection under simulated oxidative stress conditions, and (c) in the presence of 34 ng/ml transforming growth factor β1 (TGF-β1), to evaluate antifibrotic properties under simulated fibrotic conditions. Accordingly, mitochondrial dehydrogenase activity, cell proliferation, collagen production, IL-1β and IL-6 production by HDF were selected as primary outcomes.

A secondary aim of the study was to assess the antiradical activity, in terms of scavenging activity against the DPPH (2,2-di(4-tert-octylphenyl)-1-picrylhydrazyl) free radical, to evaluate whether an additional antiradical activity could contribute to cytoprotection. Accordingly, the scavenging activity against DPPH was selected as the secondary outcome.

Chemicals

Berberine and DPPH (2,2-di(4-tert-octylphenyl)-1-picrylhydrazyl, free radical), Direct Red 80 and picric acid were purchased from Sigma Aldrich (St. Louis, MO, USA). Stylopine was purchased from LGC Standards (Milan, Italy), Canadine was purchased from Ambinter (Orléans, France), Dihydroberberine was purchased from AvaChem Scientific (San Antonio, TX, USA), Coptisine was purchased from Waterstone Technology (Carmel, IN, USA), TGF-β1 was purchased from Space Import-Export (Milan, Italy).

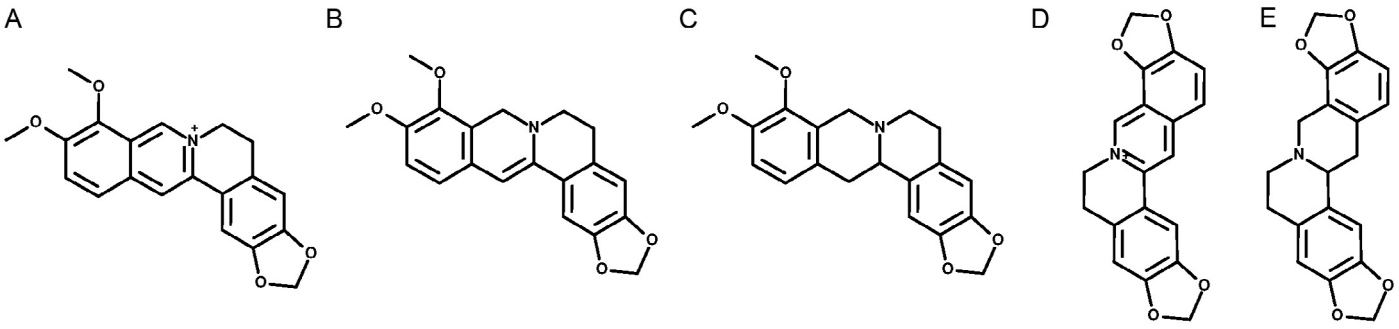

Fig. 1. Chemical structures of the five proto-berberine alkaloids investigated in the present study: berberine (A), dihydroberberine (B), canadine (C), coptisine (D) and stylopine (E).