Original Article

Understanding the ultrastructural aspects of berberine-induced skin-darkening activity in the toad, *Bufo melanostictus*, melanophores

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**A B S T R A C T**

Berberine is an active compound of *Berberis vulgaris* (Daruhaldi) with known multiple pharmacological activities, including antimicrobial, antiviral, anti-inflammatory, cholesterol-lowering, and anticancer effects. The present work aimed to study the ultrastructural effects of berberine to determine its skin-darkening potential using *Bufo melanostictus* melanophores, which has not been done to date. Light and electron microscopic analysis of isolated dorsal skin melanophores of *B. melanostictus* has been done after treatment with various concentrations of berberine, along with specific antagonists and agonists of β-adrenoceptors in order to explore the mechanism of action of berberine-induced skin darkening. The results showed that the number of melanophores with melanin-loaded dendrites increased in the subepidermal layer significantly in berberine-treated skin pieces in a dose-dependent manner leading to skin darkening. Highly electron-dense melanosomes of Stage IV increased considerably due to the enhanced process of melanization. These effects were found to be antagonized by propranolol, and were also found to be highly potentiated by isoprenaline, which is a specific β-adrenoceptor agonist. The findings show that berberine possesses a skin-darkening potential and could be used as a safe melanogenic agent for the treatment of hypopigmentation disorders or vitiligo.

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1. Introduction

Pigmentation in vertebrates is the result of the synthesis and distribution of melanin in the skin, hair bulbs, and eyes. Melanin is produced by black, dendritic pigment cells known as melanocytes within specialized cytoplasmic organelles known as melanosomes [1]. The movement of melanin granules within the melanocytes is most effectively displayed in lower vertebrates. In many of these animals, the cutaneous melanocytes play an important role during rapid changes of skin color. Matthews [2] has revealed that the postulate of contraction or rounding up of melanocytes during the lightening and sprouting of dendrites during darkening was, in reality, the visible results of translocation of pigment granules inside the relatively fixed cellular outline. This observation got confirmed by electron microscopy [3,4].

The effects of various adrenergic, cholinergic, histaminergic, and serotonergic drugs [5–13] have been studied on lower-vertebrate melanocytes, since they are a disguised type of smooth-muscle cell, and the mechanism of skin darkening is analogous to contraction and relaxation of the smooth-muscle cells of higher animals [14,15]. The pharmacological aspects of the intracellular...
displacement of melanosomes in vertebrate melanophores or melanocytes have rarely been studied at ultrastructural level, keeping in view the therapeutic approach toward melanin dysfunctions, like vitiligo/leukoderma, hypopigmentation, etc. The comparative drug-receptor studies on the melanocytes of vertebrates from a pharmacological point of view, as well as the ultrastructural details of vertebrate melanophores/melanocytes, are lacking.

With the emergence of drug resistance and the cost-effectiveness of synthetic drugs, there is a growing interest in natural products [16]. Berberine is a plant alkaloid with a long history of medicinal use in both Ayurvedic and Chinese medicine. It has been extensively investigated and has been found to possess a wide variety of pharmacological and biological activities, such as antimicrobial, antihelminthic, anti-inflammatory, and antioxidantive [17]. Ko et al. [18] examined the vasorelaxant effect of berberine in rat isolated mesenteric arteries. These findings suggested that berberine could act at vascular smooth muscles to induce relaxation. Except this, no such studies have been made to examine the effects of berberine on vertebrate pigment cells.

Recently, we have demonstrated that plant extracts of *Psoralea corylifolia*, *Ammi majus*, *Withania somnifera*, and *Ficus* species stimulate melanogenesis in various melanophore models of animals, like fishes, amphibians, and reptiles [19–24]. However, the mechanism of the

**Fig. 1.** Light microscopic photomicrograph of dorsal skin melanophores of toad showing (A) control condition of isolated dorsal skin melanophores showing neither aggregation nor dispersion by pretreatment with adrenaline (2 × 10^{-6} g/mL), 100×; (B) dispersal condition of adenalized skin melanophores by berberine at the concentration of 6.4 × 10^{-5} g/mL, 100×; (C) melanin-dispersing blocking effect of β-adrenoceptor antagonist, propranolol (4 × 10^{-7} g/mL), 100×; and (D) highly dispersed condition of skin melanophores potentiated by isoprenaline (4 × 10^{-7} g/mL), a specific agonist of β-adrenergic receptors, 100×.

**Fig. 2.** Showing the dose–response curve for the melanophore-dispersal effect of berberine (♦, closed clubs) on adenalized melanophores, with the complete blocking of the dispersal effect by a specific antagonist of β-adrenergic receptors, such as propranolol (4 × 10^{-7} g/mL, ■ and ○), and the potentiating effect on the dispersion response of berberine by a β-adrenoceptor agonist (i.e., isoprenaline) (4 × 10^{-7} g/mL, ▲). RI signifies the mean melanophore size index after the reimmersion of dorsal skin melanophores in normal amphibian Ringer saline after repeated washings. Abscisae: doses of berberine and antagonists in g/mL. Ordinate: responses of melanophores (mean melanophore size index). Vertical bars represent the standard error of mean.
induced melanogenesis at the cellular or ultrastructural level is not properly understood. Also, it is not at all known how plant extracts/phytochemicals, which are precursors of most pharmacological compositions, repopulate or regenerate the vertebrate pigment cells in vitro as well as in vivo, including human beings at the ultrastructural level. Hence, in the present study, an attempt has been made to investigate the effects of berberine on the melanophores of *Bufo melanostictus* by means of transmission electron microscopy (TEM) in order to better understand the mechanistic changes that occur in melanocytes at the cellular level during the process of induced skin darkening. The present work also provides an excellent opportunity to understand the “drug–cell model” interaction at the cellular level, which will help in understanding the mechanisms of pigmentary disorders.

### 2. Materials and methods

For the present study, the compound, berberine chloride hydrate (97% high-performance liquid chromatography), was purchased from Alfa Aesar, Johnson Matthey Co., Heysham, England. Glutaraldehyde 25% electron-microscopy grade was purchased from SERVA Electrophoresis GmbH, Heidelberg, Germany. Paraformaldehyde (catalog number UN2213) was purchased from SERVA Electrophoresis GmbH. Metaprolol was purchased from AstraZeneca Pharma Ltd., Bangalore, India. Propranolol was purchased from Cipla, Mumbai, India. Isoprenaline was purchased from SGPharma, Mumbai, India. Adrenaline was purchased from Neon Laboratories Ltd., Mumbai, India. Potassium chloride and sodium chloride were purchased from Loba Chemie Pvt. Ltd. (Mumbai, India). Sodium bicarbonate was purchased from RFCL (RANKEM) Ltd., New Delhi, India, and calcium chloride from Fisher Scientific, Mumbai, India. Other reagents were of analytical grade.

#### 2.1. In vitro dorsal skin preparation

Indian toads, *B. melanostictus*, were collected from the local area of Bhopal, Madhya Pradesh during the rainy season, and kept in large aquaria with a normal 12 h/d photoperiod. Prior to the experiments, *B. melanostictus* were allowed to acclimatize to laboratory conditions for 2 days. During the acclimatization period, the toads were
regularly fed with live snails and small insects. Diseased, injured, or lethargic *B. melanostictus* were removed, and only active, uniformly sized animals were used. For *in vitro* studies, the dorsal skin was peeled away from the decapitated toad, and a series of nearly 1-mm-by-1-mm-diameter dorsal skin pieces were cut out with a pair of sharpened steel scissors. The pieces were kept in 10 mL of amphibian Ringer saline (ARS) containing 111 mM of sodium chloride, 2 mM potassium chloride, 1 mM calcium chloride, and 2 mM sodium hydroxide in 100 mL of double distilled water at pH 7.4 in small Petri dishes, and they were equilibrated in saline medium for 15–20 min with frequent stirring. The dorsal skin pieces of the *B. melanostictus*, containing about 30–50 melanophores in the control ARS, had the tendency of dispersion within a period of 30–60 min; hence, they were brought to the intermediate state of neither aggregation nor dispersion by using a very low concentration of adrenaline (2 × 10−8 g/mL) as per the modified standard methods of Ali et al. [11], Ryan et al. [12], and Ali et al. [13]. This facilitated the accurate measurement of the dispersal responses of the melanophores induced by the berberine. The responses of the control as well as of those melanophores that were incubated in 10 mL ARS containing various concentrations of berberine (1 × 10−6 g/mL to 6.4 × 10−5 g/mL), along with their specific antagonists and agonists, were measured accordingly to the method of Bhattacharya et al. [25]. This is a modified method of Hogben and Slome [26], in which the actual diameter (length × breadth) of 10 randomly selected melanophores was recorded using a Leitz ocular micrometer, calibrated previously with 10 × 10 magnification. The value thus obtained was then multiplied by the unit of the micrometer, which was 15 μm. Thereafter, the arithmetical mean was calculated. This was the mean melanophore size index (MMSI) expressed in terms of micrometers.

The Ethical Committee for Animal Experimentation and Research, Saifia College of Science, Bhopal, India certified the use of animals (approval number SSC/06-06-22/; dated October 26, 2006). The research work of the institute is done in strict compliance with the Guidelines for Use of Laboratory Animals in Medical Colleges (2001) of the Indian Council of Medical Research, as well as with the Breeding of and Experiments on Animals Amendment Rules (2001) and the Prevention of Cruelty to Animals Act (1966).

### 2.2. TEM

Small pieces in selected concentrations of berberine were fixed in 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M sodium-phosphate buffer (pH 7.3) for 12 h at 4 °C. After washing in buffer, the skin pieces were postfixed in 1% OsO4 for 1 h at 4 °C. The samples were dehydrated in an ascending grade of acetone, and then infiltrated and embedded in araldite CY 212 (TAAB, West Berkshire, UK). Thick sections (1 μm) were cut with an ultramicrotome, mounted onto glass slides, stained with aqueous toluidine blue, and observed under a light microscope for gross observation of the area and quality of the tissue fixation. For the electron-microscope examination, thin sections of gray–silver color interference (70–80 nm) were cut and mounted onto 300-mesh copper grids. The sections were stained with alcoholic uranyl acetate and alkaline lead citrate, washed gently with distilled water, and observed under a Morgagni 268D transmission electron microscope (FEI Company, Eindhoven, The Netherlands) at an operating voltage of 80 kV. Images were digitally acquired by using a charge-coupled-device camera (Megaview III; FEI Company) attached to the microscope. TEM was done at All India Institute of Medical Science, New Delhi, India.

### 2.3. Statistical analysis

Statistical data analyses are presented as mean ± standard error of the mean; *n = 7* represents the number of dose concentrations (treated) used for a particular experiment. Comparisons were made between the treated and control groups by use of the Student’s *t*-test. All data were analyzed using GraphPad Prism software (San Diego, CA). A value of *p < 0.05* indicates a statistically significant difference.

### 3. Results

#### 3.1. Light and electron microscopic observations of the control dorsal skin melanophores of *B. melanostictus* in ARS

Light microscopic control vertical sections of the dorsal skin of *B. melanostictus* showed a moderate degree of pigmentation where melanophores were present in neither aggregated nor dispersed state. The MMSIs of
such control melanophores were recorded as 3.86 ± 0.19 (Figs. 1A and 2). In moderately pigmented control skin pieces, these pigmented spots were found to be scattered throughout the dorsal surface. One distinct subepidermal pigment cell layer was observed. Occasionally, epidermal glands occur interrupting the epidermal layers, although epidermal melanophores are rare. In the moderately pigmented parts of the dorsal skin, melanophores were present, whereas xanthophores and irridiophores were absent (Fig. 3A). The darkness of the skin also seemed to depend on the presence of melanophores in the epidermal, subepidermal, and dermal layers. Toad melanophores are organized in two distinct layers: one subepidermal melanophore layer, which remains present closely below the epidermis, while the other remains scattered in the loose dermal matrix of the skin.

Electron microscopic observations of the control dorsal skin melanophores of toads have thrown light on remarkably consistent fine structural features of these melanosome-synthesizing cells. Subepidermal melanophores contain a round nucleus, which possesses a coarsely granulated nucleoplasm. The nucleus of the melanophores remains surrounded by numerous melanosomes in various stages of maturation (Fig. 3B).

Mitochondria containing dense particles in their matrices and vacuolar endoplasmic reticulum were found to be dispersed between the melanosomes (Fig. 3C). The majority of the melanosomes are ovoid and are surrounded by unit limiting membranes. Internally, many melanosomes exhibit a thin concentric electron-lucent layer between the central and cortical zones, while others appear to contain slightly less electron-dense cores surrounded by denser cortices (Fig. 4A and B).

3.2. Effect of berberine on the isolated dorsal skin melanophores of B. melanostictus

Berberine was added in the incubating medium of normal toad-skin melanophores in a concentration range of 1 × 10⁻⁸ g/mL to 6.4 × 10⁻⁵ g/mL. It was found that berberine per se induced a powerful, dose-dependent pigment dispersion in all the skin melanophores of toad B. melanostictus in comparison to the controls, which had made the berberine-treated skin to appear dark (Fig. 1B). The maximum concentration of 6.4 × 10⁻⁵ g/mL of berberine resulted in a complete dispersion of all the melanophores of the toad, where the MMSI increased from a control level of 3.86 ± 0.19 to 8.89 ± 0.07 (Fig. 2). This increased
pigmentation was seen in the occurrence of subepidermal cell melanophores and dermal melanophores, as revealed by light microscopy (Fig. 5A and B). The melanophores in these layers showed increased dendritic processes after treatment with different concentrations of berberine (Fig. 1B). Cross sections of dendrite processes were extremely complex in shape, making it difficult to determine the shape of the melanophores, suggesting an extensive ramification of the dendritic melanophores. Most of the melanin visible in the micrographs at suprabasal levels, in fact, was found in these dendritic processes. Dermal melanophores, on the other hand, were commonly observed scattered in loose dermal matrix (Fig. 5A and B).

Ultrastructural observations revealed that the subepidermal melanophores of the toad contain membrane-bound electron-dense melanosomes. A large number of vesicles occupy one side of the perinuclear-cytoplasm intensely electron-dense oval melanosomes, which were evenly distributed in the peripheral region (Fig. 5C). Few premelanosomes were observed in the cytoplasm as berberine treatment had induced the process of melanization of the premelanosomes. As a result of this, Stage II premelanosomes got converted into electron-lucent Stage III premelanosomes. At a higher concentration of berberine (6.4 x 10^{-5} g/mL), a complete melanization of the melanosomes occurred, which led to a complete electron-dense appearance of melanosomes (Fig. 6A and B). In the relatively dense cytoplasm around the nucleus, the mitochondria remained aligned parallel to the nuclear envelope. The remaining cytoplasmic areas contained smooth-surfaced vacuolar elements of the endoplasmic reticulum, collagen fibers, and microtubules, randomly oriented between the organelles, as shown in Fig. 6A and C.

3.3. Effect of berberine on the isolated dorsal skin melanophores of B. melanostictus pretreated with β-adrenergic agonists

The melanin-dispersal effect of berberine was studied after the pretreatment of melanophores by the β-adrenergic antagonist, propranolol. It was found that propranolol (4 x 10^{-7} g/mL), completely blocked the powerful per se melanophore-dispersal effects of berberine (Fig. 7A and B). A significant inhibition of the extent of pigment dispersion was seen in the melanophore preparation preexposed to propranolol (Fig. 1C). The MMSI at the highest concentration of 6.4 x 10^{-5} g/mL berberine was recorded as 3.32 ± 0.23, which, without propranolol, was 3.86 ± 0.19 (Fig. 2). The light microscopy of the antagonist-treated dorsal skin revealed that the degree of pigmentation had reduced considerably as the melanophores decreased in size. Few pigment cells were seen in between the subepidermal and dermal layers of the skin (Fig. 7B). The propranolol treatment reversed the effect of an even higher concentration of berberine (6.4 x 10^{-5} g/mL), as it induced a decrease in the dendritic process of the melanophores. The diameter of the pigment cells also decreased (Fig. 1C). Dermal melanophores per unit area also decreased as compared with the control pieces of skin due to the blocking effect of the antagonist. The electron microscopic observation also showed that the melanophores had irregularly indented nuclei in the subepidermis (Fig. 7D). Only stages II and III of oval premelanosomes containing a fibrillar, granular, and partially melanized central melanin body were frequently observed (Fig. 7E), which were non-electron dense in appearance (Fig. 7C).

3.4. Effect of the berberine-treated isolated dorsal skin melanophores of B. melanostictus pretreated with β-adrenergic agonists

The dorsal skin pieces containing melanophores of B. melanostictus were subjected to isoprenaline treatment with a constant dose of 4 x 10^{-7} g/mL, after which they were further treated with berberine per se. The skin-darkening effect of berberine was found to be potentiated by the β-adrenoceptor agonist, isoprenaline (Fig. 1D). The MMSI of this case read as 9.72 ± 0.53 at the highest concentration of berberine (6.4 x 10^{-5}), which is three times higher than the control value of 3.86 ± 0.19 (Fig. 2). Semithin sections of the dorsal skin of B. melanostictus were viewed under a research light microscope (Quasmo Microscopes, Haryana, India). The isoprenaline treatment potentiated the effect of berberine, as it led to increase
the degree of pigmentation. An increased number of melanophore dendrites present between the subepidermis and the dermis were seen per unit area, which consequently resulted in the darkening of the toad skin. The melanophores remained randomly scattered horizontally, and the number of scattered dermal melanophores got increased in the loose dermal matrix (Fig. 8A). Fine structural observations revealed that the nucleus of the melanophores had become more prominent, round, and was filled with granular electron-opaque nucleoplasm. A large number of fully developed Golgi complex associated vesicles, and rough and vacuolar smooth endoplasmic reticulum were also observed. Characteristic numbers of highly electron-dense oval melanosomes were clearly visible in the cytoplasm of the subepidermal melanophores (Fig. 8B and C).

4. Discussion

The light-microscope examination of the semithin vertical sections of the dorsal skin melanophores of *Bufo melanostictus* indicated that melanophores are present dominantly in the subepidermis, called as subepidermal melanophores. Dermal melanophores in this species have been found in very less number and remain situated just below the subepidermal layer, although some melanophores occur deeper in the dermis, as revealed by Kijewskas et al. [27] in the Atlantic cod, *Gadus morhua*. We have examined the effects of berberine *per se*, and with its specific antagonists and agonists on toad-skin subepidermal melanophores using the electron microscope, and found that berberine stimulated the process of melanization, as well as increased the number of melanin-loaded...
dendrites of the subepidermal melanophores of the toad, *Bufo melanostictus*. We have observed that melanin deposition occurs in the melanosomes of Stage III; hence, the number of mature melanosomes of Stage IV increased rapidly, which is in accordance with the work of Seiji et al. [28], who reported that the melanosomes are the sole site of melanin synthesis in mammalian melanocytes. The distinction between stages III and IV melanosomes appears to be simply in the content of melanin-pigment deposition [29,30]. The appearance of electron-dense melanosomes under the influence of berberine indicates that this phytocompound elicits the melanization of melanosomes inside the subepidermal melanophores. The association of these melanin-containing granules with the Golgi complex shows that they are developed from the Golgi vesicles, as described by Seiji et al. [31]. Further, the fully developed toad melanosomes were found to be markedly electron-opaque oval bodies surrounded by unit limiting membrane quite similar to the melanosomes present in the epidermis of humans, as described by Hori et al. [32], Spencer et al. [33], and Iyengar [34].

In the present study, it has been found that the skin-darkening effect of berberine *per se* was blocked by propranolol, which is a specific antagonist of β-adrenergic receptors. It appears that propranolol blocked the maturation of premelanosomes as a result of which no melanosomes of Stage IV could be seen. At this junction, the structure of the late premelanosomes of the toad subepidermal melanophores without melanization suggests that propranolol pretreatment resulted in the complete arrest of the process of melanin deposition on the individual fibrils of a protein framework present within immature melanosomes (Stage II), which is in accordance to the work of Fukuzawa [30], in which phenyl thiourea exerts a similar effect in the tail melanophores of *Xenopus laevis* tadpole. Premelanosomes containing protein fibrous matrices similar in appearance to those asserted in *B. melanostictus* also have been reported by Khoo et al. [4] within the melanophores of the Siamese fighting fish (*Betta splendens*). We also reported here that, in contrast to the abundance of premelanosomes within the sub-epidermal melanophores, the dermal melanophores of toad contains comparatively lesser number of premelanosomes whose internal structure seems to be similar to those of the sub-epidermal melanophores.
It has been observed that the physiologically significant dose-related berberine-induced melanization of toad-skin subepidermal melanophores was further enhanced by the specific agonist of β-adrenoceptors (i.e., isoprenaline), as a result of which Stage VI melanosomes were seen predominantly scattered throughout the subepidermal matrix. The other fine structural changes described previously for the berberine-darkened cells were accentuated due to isoprenaline pretreatment. It has been well documented that melanin synthesis is enhanced by numerous agents, such as UV radiation, α-melanocyte-stimulating hormone, forskolin, and isobutyl methyl xanthine [35–39]. Very few of these are used as skin enhancers because of their ineffectiveness or undesirable side effects. We have demonstrated in the present study that berberine, which is present in the roots of Berberis vulgaris, is one of those agents that can be used to effectively enhance skin darkening without any unwanted side effects. The results presented here provide evidence that berberine is able to induce skin darkening and increased dendricty in the dorsal skin melanophores of B. melanostictus in a dose-dependent manner. The physiologically significant melanin-dispersal response of berberine was found to be completely abolished by propranolol, which is a specific β-adrenergic-receptor antagonist. The increase in the number of active melanocytes was accelerated by pretreating the dorsal skin of toad with isoprenaline, which is a specific β-adrenoceptor agonist. As far as is known, this is the first report of its kind where berberine has been found to stimulate the dominantly present β-adrenergic receptors in the pigment cell system of toads, leading to skin darkening. These findings are in agreement with those of Peter et al. [40] and Chaudhari et al. [41], who showed that melanin dispersion in Hemidactylus flaviviridis and Channa punctatus, respectively, was found to be mediated by the dominantly present β-adrenoceptors. However, there are no reports in the literature showing the activation of β-adrenergic receptors by berberine in any of thepigmented cells, including the melanophores. In this regard, the work of Neto [42] is worth mentioning, as he has demonstrated the involvement of β-adrenoceptor in the berberine-mediated regulation of the transmembrane electric potential on canine cardiac Purkinje and ventricular muscle fibers, and on rabbit atrial fibers. Chiou et al. [43] have indicated that berberine vasodilates the rat mesenteric artery by directly blocking the release of Ca2+ from internal stores. In view of earlier pieces of work, if the present data are interpreted, it seems that berberine induces the dispersion of melanin pigment, leading to skin darkening in a similar fashion.

These findings offer better understanding of the ultrastructural changes that occurred in melanophores following berberine treatment. Also, we have attempted to explore the pharmacological effect of berberine with regard to the mechanism involved in the melanization of melanosomes within the melanophores at the cellular level, using the ultrastructural analysis, which was wanting so far. The data also suggest the evolutionary significance of lower-vertebrate melanophores with that of more evolved mammalian melanocytes from the point of view of β-adrenergic-receptor involvement in stimulating melanosomes at the ultrastructural level.

Conflicts of interest

The authors have no conflict of interest to declare.

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