Journal of Photochemistry & Photobiology, B: Biology 156 (2016) 61–68

Contents lists available at ScienceDirect

Journal of Photochemistry & Photobiology, B: Biology

journal homepage: www.elsevier.com/locate/jphotobiol

Phototoxic potential of silymarin and its bioactive components

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ARTICLE INFO

Article history:
Received 16 November 2015
Received in revised form 18 January 2016
Accepted 25 January 2016
Available online 26 January 2016

Keywords:
Flavonolignans
Phototoxicity
3T3 NRU
Balb/c 3T3
HaCaT
Primary skin cells

ABSTRACT

Silymarin, a standardized extract of the seeds of the milk thistle (*Silybum marianum*) and its major component, silybin, is now used as an active component in a broad spectrum of dietary supplements, cosmetics and dermatological preparations. However, despite its use in skin products, there are no published data to exclude its phototoxic potential. The primary purpose of this study was to examine the phototoxicity of silymarin and its flavonolignans, silybin, isosilybin, silychristin, silydianin and 2,3-dehydrosilybin by validated 3T3 NRU assay. Further, we compared the validated biological system Balc/c 3T3 cell line with other cell models, particularly normal human dermal fibroblasts (NHDF), normal human epidermal keratinocytes (NHEK) and the human keratinocyte cell line (HaCaT). The results showed that silymarin and the flavonolignans silybin, isosilybin, silychristin and silydianin had no phototoxicity towards any of the cells used. In contrast, 2,3-dehydrosilybin was identified as a compound with phototoxic potential. Further study is needed to evaluate the health risks associated with 2,3-dehydrosilybin use in skin preparations.

1. Introduction

Many different classes of drugs as well as ingredients in personal care products have been found to be activated by solar radiation and stimulate a phototoxic response in the skin [1]. Photoactivated molecules may elicit harmful effects including phototoxicity (*e.g.* erythema, oedema, pigmentary alterations), photoallergy and photocarcinogenity [2]. For this reason, evaluation of phototoxic hazard is usually required by law in most EU countries for ingredients/products for pharmaceutical use including personal care products particularly when used on body areas which may be exposed to sunlight and the ingredients absorb UV and visible light in the region of 290–700 nm [2,3].

Milk thistle (*Silybum marianum* L. Gaertner; Asteraceae) is one of the oldest known herbal plants that have been widely used in traditional European medicine for over two thousand years, especially for treating liver disorders and to protect the liver against poisoning from chemical and environmental toxins [4]. A multicomponent extract from the seeds of *S. marianum* is called silymarin (SM) and consists of at least seven flavonolignans. The extract is available from several major producers, each with its own standard composition and this varies among producers depending on a variety of growing conditions [5]. The main polyphenolic component of SM is the flavonolignan silybin (SB; approximately 50–70%) that is also biologically the most active constituent. Other SM components present in considerable amounts include flavonolignans isosilybin (ISB; about 5%), silychristin (SC; about 20%), silydianin (SD; about 10%), 2,3-dehydrosilybin (DHSB) and flavonoids taxifolin (dihydroquercetin) and quercetin [4]. Experimental findings showed excellent antioxidant activity, modulation of the immune system and various signalling pathways for both SM and SB. Low short- and long-term toxicity, favourable pharmacokinetics, detoxifying, preventive and regenerative effects and minimal side effects SM very attractive therapeutic application. Curative properties of SM and SB have been studied in the treatment of Alzheimer's disease, Parkinson's disease, sepsis, osteoporosis, diabetes, cholestasis or hypercholesterolemia [6]. Beneficial effects of SM and SB have also been shown on skin carcinogenesis induced chemically or by chronic exposure to UVB light [7], on skin disorders such as contact dermatitis [8], skin hyperpigmentation (melasma) [9], radiodermatitis in oncologic patients undergoing radiotherapy [10]. For this reason, SM and SB are used as active components in a broad spectrum of dietary supplements, cosmetics and dermatological preparations. In connection with the latter, no study to our knowledge has been published to exclude the phototoxic potential of SB or SM.

As animal use for cosmetic products and ingredient testing is banned in Europe, for identifying phototoxic hazards an *in vitro* method, 3T3 Neutral Red Uptake Phototoxicity Test (3T3 NRU) has been validated. This method uses the mouse fibroblast cell line Balb/c 3T3 clone A31 [11]. Due to its sensitivity, specificity and robustness, the 3T3 NRU is considered the core test besides additional tools such as reconstructed human skin models or the human *in vivo* photopatch test. However, the use of monolayer cannot reflect the real situation in human skin

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http://dx.doi.org/10.1016/j.jphotobiol.2016.01.011
1011-1344/

due to skin morphology. The absorption and skin penetration of compounds are affected especially by the outer epidermal layer (*stratum corneum*) that represents a fundamental barrier not only for chemicals. This very important attribute of human skin limits the ability of xenobiotics and hazardous substances from entering the body and cannot be detected when monolayer is utilized [12]. Animal cells such as Balb/c 3T3 probably differ in response to toxins because the shorter lifespan of animals means the cytoprotective and regenerative pathways in animal cells are not as powerful as human ones. Further, Balb/c 3T3 cells are embryo cells [13] that may be more sensitive to photoactivated chemicals compared to human skin cells that are predetermined to form a barrier against environmental assault including chemicals. Some studies however suggest that a positive phototoxic result in the 3T3 NRU may not correlate with results on reconstructed human skin and/or human skin *in vivo* [14–16] and may not necessarily represent a hazard to human skin. Therefore, follow-up tests employing human skin cells need to be developed.

The primary purpose of this study was to examine the phototoxic potential of SM including its major flavonolignan SB and minor flavonolignans (ISB, SC, SD and DHSB) using the Balb/c 3T3 cell line, a validated biological system. Further, we decided to compare the ability of human skin cells, particularly normal human dermal fibroblasts (NHDF) and normal human epidermal keratinocytes (NHEK) and the human keratinocyte cell line (HaCaT) to evaluate the phototoxic potential of selected flavonolignans and SM in relation to the strong phototoxic substance, chlorpromazine (CPZ). NHDF were employed as an alternative to mouse cell line Balb/c 3T3 to assess whether individual difference between human donors significantly affects the results of 3T3 NRU. HaCaT were employed as they are frequently used as a suitable alternative to NHEK [17–19] as well as intentionally to detect the variation in results that may emerge as a result of donor variations in primary NHEK.

2. Material and Methods

2.1. Chemicals

Keratinocyte basal medium-2 (KBM-2), KGM™-2 SingleQuots™, EpiLife® Medium and Human Keratinocyte Growth Supplement Kit were received from Life Technologies s.r.o. (Czech Republic). Dulbecco's modified Eagle's medium (DMEM), Ham-F12 Nutrient Mixture, heat-inactivated foetal calf serum (FCS), heat-inactivated newborn calf serum (NCS), stabilized penicillin–streptomycin solution, amphotericin B, hydrocortisone, adenine, insulin, epidermal growth factor, 3,3′,5-triiod-L-thyronin, trypsin, ampicillin, trypsin–EDTA (0.25%), dimethyl sulfoxide (DMSO), neutral red (NR), crystal violet and other chemicals were from Sigma-Aldrich (Czech Republic).

2.2. Test Compounds

SM (batch 17306S/089) containing 71% of flavonolignans and silybin (SB; batch 120 meter 692; purity 98%; natural mixture of diastereomers *ca.* 1:1) were kindly provided by Teva Pharmaceutical Industries Ltd. (Opava, Czech Republic). ISB (natural mixture of diastereomers *ca.* 95:5), SC (natural mixture of diastereomers *ca.* 9:1) and SD were isolated from SM by Sephadex LH-20 column chromatography as described previously [20]. DHSB was prepared as described by *Gažák* et al. [21]. Briefly, SB was refluxed in acetic acid with iodine and potassium acetate and precipitated with water. Acidic hydrolysis and crystallization with ethanol afforded DHSB in a good yield and purity. Chlorpromazine hydrochloride (CPZ) was purchased from Sigma Aldrich. Chemical structures of the flavonolignans are shown in Fig. 1.

2.3. Source of Radiation

The UV light source for phototoxicity testing was a solar simulator SOL 500 with a spectral range (295–3000 nm) corresponding to natural sunlight. The simulator was equipped with a *H1* filter transmitting wavelengths of 315–380 nm. The UVA output before each experiment was measured by an UVA-meter (Dr. Hönle UV Technology, Germany).

2.4. Cell Culture

Mouse fibroblasts Balb/c 3T3 cell line (clone A31, No 86110101) was purchased from the European Collection of Cell Cultures (Salisbury, United Kingdom). The cells were grown in DMEM supplemented with FCS (5%; *v*/*v*) and NCS (5%; *v*/*v*), streptomycin (100 U/ml) and penicillin (0.1 mg/ml in a humidified atmosphere with CO_2 (5%; *v*/*v*) at 37 °C.

HaCaT, a spontaneously transformed human epithelial cell line developed by P. Boukamp and N. Fusenig [22], were purchased from CLS (Eppelheim, Germany). Cells were cultured in DMEM supplemented with FCS (10%; *v*/*v*), streptomycin (100 U/ml), penicillin (0.1 mg/ml) in a humidified atmosphere with CO_2 (5%; *v*/*v*) at 37 °C.

NHDF and NHEK were harvested from the skin of medically healthy adult donors. The tissue specimens were obtained from patients undergoing plastic surgery at the Department of Plastic and Aesthetic Surgery (University Hospital in Olomouc). The tissue acquisition protocol adhered to the requirements of the Ethics Committee of the University Hospital in Olomouc and Faculty of Medicine and Dentistry, Palacký University in Olomouc. All patients had signed written informed consent. Before use the tissues were washed three times in phosphate buffered saline (PBS) containing antibiotics (penicillin (500 mg/ml), streptomycin (500 U/ml) and amphotericin B (1.25 mg/ml)). NHEK were isolated according to protocol of *Minner* et al. [23] with some modifications [24]. Briefly, the tissue was cut into pieces and incubated in trypsin solution (0.5%) overnight at 4 °C. The epidermis and dermis were then separated and the epidermal tissue was placed in the KBM-2 supplemented with KGM™-2 SingleQuots™ and FCS (2%; *v*/*v*). The epidermal cells were mechanically dissociated. The cell suspension was filtered through a cell strainer and centrifuged (5 min, 120 *g*, 4 °C). The pellet was resuspended in KBM-2, transferred into 75 cm^2 cultivation flasks and cells were then grown in humidified atmosphere with CO_2 (5%; *v*/*v*) at 37 °C for 3 days. KBM-2 was then changed by the growth EpiLife® medium supplemented with Human Keratinocyte Growth Supplement Kit, penicillin (100 mg/ml), streptomycin (100 mg/l) and ampicillin (250 μg/ml). Keratinocytes were used at the 3^{th} passage.

In the case of NHDF, the tissue was cut into pieces of approximately 1 × 1 cm, placed in Petri dishes and cultured in the mixture of DMEM and Ham's F12 Nutrient Mixture (1:3) supplemented with FCS (10%; *v*/*v*), penicillin (100 mg/ml), streptomycin (100 U/ml), amphotericin B (0.125 mg/ml), hydrocortisone (0.8 μg/ml), adenine (24 μg/ml), insulin (0.12 U/ml), epidermal growth factor (1 ng/ml) and 3,3′,5-triiod-L-thyronin (0.136 μg/ml). The skin fragments were incubated in a humidified atmosphere with CO_2 (5%; *v*/*v*) at 37 °C. The medium was changed weekly until the fibroblasts reached confluence. After 2–3 weeks cells were trypsinized and transferred into 75 cm^2 cultivation flasks. Cells were then grown in DMEM supplemented with FCS (10%; *v*/*v*), penicillin (100 mg/ml) and streptomycin (100 U/ml) in a humidified atmosphere with CO_2 (5%; *v*/*v*) at 37 °C. Fibroblasts were used between the 2^{nd} and 4^{th} passage.

For experiments, cells were seeded onto 96-well plates at the density of 0.5×10^5 cells/cm^2 (Balb/c 3T3 and NHDF), 0.625×10^5 cells/cm^2 (NHEK) or 1×10^5 cells/cm^2 (HaCaT).

2.5. Determination of Phototoxicity

Twenty-four hours after seeding, cells morphology was checked under an inverted phase-contrast microscope (Olympus CK2 TR;