Fitoterapia 110 (2016) 1–7

Contents lists available at ScienceDirect

Fitoterapia

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

Anticancer activity of glucomoringin isothiocyanate in human malignant astrocytoma cells

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ARTICLE INFO

Article history:
Received 30 December 2015
Received in revised form 11 February 2016
Accepted 12 February 2016
Available online 13 February 2016

Keywords:
Isothiocyanates
Glioblastoma multiforme
Glucomoringin
Apoptosis
Oxidative stress
Anti-tumor effects
Moringa oleifera

ABSTRACT

Isothiocyanates (ITCs) released from their glucosinolate precursors have been shown to inhibit tumorigenesis and they have received significant attention as potential chemotherapeutic agents against cancer. Astrocytoma grade IV is the most frequent and most malignant primary brain tumor in adults without any curative treatment. New therapeutic drugs are therefore urgently required. In the present study, we investigated the *in vitro* antitumor activity of the glycosylated isothiocyanate moringin [4-(α-L-rhamnopyranosyloxy)benzyl isothiocyanate] produced from quantitative myrosinase-induced hydrolysis of glucomoringin (GMG) under neutral pH value. We have evaluated the potency of moringin on apoptosis induction and cell death in human astrocytoma grade IV CCF-STTG1 cells. Moringin showed to be effective in inducing apoptosis through p53 and Bax activation and Bcl-2 inhibition. In addition, oxidative stress related Nrf2 transcription factor and its upstream regulator CK2 alpha expressions were modulated at higher doses, which indicated the involvement of oxidative stress-mediated apoptosis induced by moringin. Moreover, significant reduction in 5S rRNA was noticed with moringin treatment. Our *in vitro* results demonstrated the antitumor efficacy of moringin derived from myrosinase-hydrolysis of GMG in human malignant astrocytoma cells.

1. Introduction

Isothiocyanates (ITCs) are a well-established group of natural products, which are not produced as such by the plant, but rather released after cell damage by the enzymatic action of myrosinase (β-thioglucoside glucohydrolase; E.C. 3.2.1.147) on their glucosinolate (GLs) precursors [1,2]. To date, about 130 different GLs have been identified in plants of the botanical order Brassicales [3], and their breakdown products. ITCs have long been known for their key role in antitumoral activity and antiinflammatory response [4]. Observational studies have shown that consumption of GLs/ITC-rich cruciferous vegetables protects against many types of cancer in humans *via* apoptosis and the induction of cell cycle arrest, and these anticancer properties might be attributed to the presence of a high content of naturally occurring ITCs, including benzyl ITC (BITC), phenethyl ITC (PITC), allyl ITC (AITC), and sulforaphane [5]. Glucomoringin (GMG) (4-(α-L-rhamnopyranosyloxy)benzyl GL is an uncommon member of the GL family present in vegetables belonging to the family Moringaceae: *Moringa oleifera* commonly called the "horse–radish tree", is the most widely distributed species in the genus Moringa [6]. The glycosylated compound 4-(α-L-rhamnopyranosyloxy)benzyl isothiocyanate (moringin), resulting from myrosinase-hydrolysis (>99%) of GMG at neutral condition (Fig. 1A), has been shown to exhibit a broad biological activity, including effective antitumor promoting activity [7,8] and apoptosis induction in *in vitro* and *in vivo* models such as myeloma, leukemia and carcinoma [9].

Among all brain and central nervous system tumors, astrocytoma grade IV, also known as glioblastoma multiforme (GBM), is the most common primary malignant glioma in adults. It represents a highly invasive, proliferative, and aggressive cancer type [10]. Total resection of tumor tissue followed by combined therapy of temozolomide (TMZ) and radiation is the current standard treatment for GBM; however, as many as 50% of high-grade tumors are resistant to TMZ therapy and eventually recur in a vast majority of patients [11]. Therefore, novel anticancer compounds with high efficacy are urgently needed. Apoptosis plays an important role in regulating a wide range of biological events, such as cell proliferation and differentiation, and dysregulation of apoptosis is associated with a variety of diseases, including cancer and neurodegenerative diseases [12,13]. Indeed, apoptosis is the most important therapeutic mechanism by which chemotherapeutic agents kill cancer cells and eradicate tumor tissues [14,15]. Moreover, screening of phytochemicals from folk medicinal plants has been emerged as

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http://dx.doi.org/10.1016/j.fitote.2016.02.007
0367-326X/

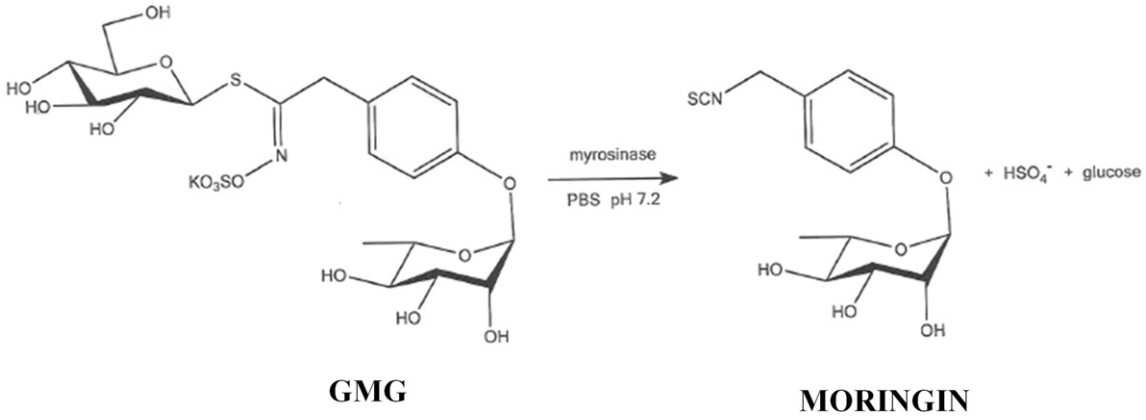

Fig. 1. Production of moringin (4-(α-L-rhamnopyranosyloxy)benzyl isothiocyanate). Reaction of myrosinase catalyzed hydrolysis of glucomoringin (GMG), purified from *Moringa oleifera* seeds, in phosphate buffered saline (PBS) solution (pH 7.2).

a promising approach against cancer [16]. Consequently, novel anticancer compounds from medicinal plants with high antitumor efficacy and low toxicity are highly desirable for cancer treatment, and they should be carefully considered during selection of an integrative approach for cancer prevention and treatment [16].

Gram-scale production of natural GMG has been recently set up starting from *M. oleifera* seeds according to a procedure developed at CREA-CIN of Bologna, Italy [9]. In the present study, we investigated the *in vitro* antitumor activity of moringin that provides the natural agent GMG-ITC in human grade IV astrocytoma CCF-STTG1 cells. We have evaluated the efficacy of moringin in inducing apoptosis and cell death.

2. Materials and methods

2.1. Isolation and purification of GMG and myrosinase

GMG was isolated from *M. oleifera* L. (fam. Moringaceae) seeds according to a method previously described [9]. In brief, GMG was purified by two sequential chromatographic steps: isolation through anion exchange chromatography, followed by gel filtration to achieve purification to homogeneity. GMG was unambiguously characterized by ^{1}H and ^{13}C NMR spectrometry and the purity was assessed by HPLC analysis of the desulfo-derivative according to the ISO 9167-1 method approved by the European Union, Commission Regulation, EEC No. 1864/90 [17], yielding GMG with a purity of 99% based on peak area value, and about 90–92% on a weight basis due to its high hygroscopic properties. The enzyme myrosinase was isolated from seeds of *Sinapis alba* L. as described by Pessina et al. [18] with some modification. The specific activity of the stock solution used in the present study was 60 U/mg of soluble protein. The enzymatic activity was 32 U/mL and the solution was stored at 4 °C in sterile saline solution at neutral pH until use. One myrosinase unit was defined as the amount of enzyme able to hydrolyze 1 μmol/min of sinigrin at pH 6.5 and 37 °C [2].

2.2. Enzyme bioactivation of GMG

GMG was dissolved in RPMI-1640 medium and cell treatment required the enzyme bioactivation of the phytochemical. The action of myrosinase enzyme (0,64 U/mL) for 15 min allowed fast production of moringin, before the cell treatment (Fig. 1). The total conversion of pure GMG into moringin was confirmed by HPLC analysis of the GMG desulfo-derivative, which allowed us to monitor the reduction of GMG until its complete disappearance in the reaction mixture [19].

2.3. Cell culture conditions and drug treatment

The human brain astrocytoma cell line CCF-STTG1 was purchased from (Centro substrati cellulari, Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia, Italy). Cells were cultured in monolayer using RPMI-1640 medium (CARLO ERBA, Italy) supplemented with 10% fetal bovine serum (FBS) (Sigma-Aldrich Co. Ltd., USA). The cells were grown in logarithmic phase at 37 °C, in a humidified atmosphere consisting of 5% CO_2 and 95% air. CCF-STTG1 cell line is a stable immortalized cancer cell line and experiments were performed with cells (70–80% confluence) not exceeding 30 passages. For drug treatment, cells were grown until 70%–80% confluence followed by overnight incubation at 37 °C with moringin at following concentration ranges (μM): 2, 4, 8, 12, 16, 24, 32, and 40. Untreated cells (CTR), GMG (40 μM) and myrosinase (MYR) treated cells were also included as controls. In order to assess the possible cytotoxic effect of moringin, human periodontal ligament tissue-derived mesenchymal stem cells (hPDLSCs) were grown in DMEM high-glucose medium (CARLO ERBA, Italy) until 70–80% confluence followed by overnight incubation at 37 °C with moringin at following concentration ranges (μM): 8, 12, 16, and 24. GMG (40 μM) and myrosinase treated cells were also included as controls. Then, the cells were either fixed or harvested for further analyses.

2.4. Immunocytochemistry

Cells on cover slips (Thermo SCIENTIFIC, Germany) were fixed with 4% paraformaldehyde at room temperature for 15 min and then washed with phosphate buffered saline (PBS, pH 7.5). Morphological changes in apoptotic cells were investigated by hematoxylin and eosin staining (H&E) using a standard protocol. For immunocytochemical staining, cells were 4% paraformaldehyde-fixed and treated with PBS-buffered 3% hydrogen peroxide (H_2O_2) at room temperature for 15 min to terminate the endogenous peroxidase activity. Then, cells were blocked with horse serum + 0.1% Triton X-100 for 20 min followed by overnight incubation at 4 °C with primary antibodies against examined proteins (anti- p53 and Bax (Santa Cruz, USA; dilution 1:100). After PBS wash, cells were incubated with biotinylated secondary antibody (1:200, Vector Laboratories, Burlingame, CA) and streptavidin ABComplex-HRP (ABC-kit from Dako, Glostrup, Denmark) followed by DAB+ (Dako) staining. Microscopy was performed using light microscopy (LEICA DM 2000 combined with LEICA ICC50 HD camera).

2.5. Protein extraction and Western blot analysis

Cells treated with moringin at different concentrations were harvested following 24 h of incubation. After washing with ice-cold PBS,