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Chemopreventive and antitumor effects of benzyl isothiocynate on HCC models: A possible role of HGF /pAkt/ STAT3 axis and VEGF

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ABSTRACT

Background: Benzyl isothiocyanate (BITC) is a member of the isothiocyanate compounds that found in cruciferous vegetables. BITC has a potential anticancer effect in different types of tumors. Few studies referred to the antineoplastic effect of BITC against HCC. The mechanism of BITC concerning retardation of HCC progression is incompletely understood.

Aim of the work: This study evaluated the role of HGF, pAkt and STAT3 in BITC induced HCC growth retardation.

Method: HCC was induced in mice using diethylnitrosamine (DEN) 75 mg/kg once a week for 4 weeks. BITC 10 and 20 mg/kg was given to mice orally each day for 10 weeks. The HCC cell lines HepG2 and Huh-7 were also used to evaluate the effect of BITC on tumor cells behavior. Immunoassay was used to detect expressions of caspase-3 activity, VEGF, MMP-2, TNF-α, HGF and pAkt. STAT3 expression was detected in liver tissues using immunohistochemical staining.

Results: BITC has a potential role in suppressing hepatic precancerous lesion progression in mice. The drug increased caspase-3 activity in tumor cells and inhibited the angiogenic marker VEGF. It also decreased the metastatic marker MMP-2. This anticancer effect of BITC was observed in DEN treated mice as well as in hepatoma cell lines. The reported antineoplastic activity was correlated with downregulation of HGF and its downstream molecules pAkt and STAT3.

Conclusion: The effect of BITC on HGF /pAkt/ STAT3 axis has a potential role in both chemopreventive and chemotherapeutic effects of BITC.

1. Introduction

Hepatocellular carcinoma (HCC) is one of the major causes of cancer related death [1]. The major challenge in HCC is the resistance to both chemotherapy and radiotherapy [2]. The needs for potent therapies as well as focusing on preventive agents are urgent.

The cruciferous vegetables, such as cabbage, broccoli and cauliflower have been suggested to have preventive effects against different types of cancers such as lung, stomach, colon, liver, esophagus, bladder, and mammary glands tumors [3,4].This anticarcinogenic efficacy has been attributed to a group of biologically active compounds called Isothiocyanates ITC [5]. Benzyl isothiocyanate (BITC) is a member of the ITC compounds that is formed as a result of glucotropaeolin hydrolysis in cruciferous vegetables [6].

Various studies referred to the importance of BITC as adjuvant therapy with traditional therapeutic protocols. BITC modulated the resistant behavior of non-small cell lung cancer to gefitinib [7]. In addition, it potentiated the apoptotic activity of gamma radiation against pancreatic cancer [8]

Accumulated data about BITC showed controversial cellular effects. Some evidences referred to the genotoxic effect of BITC as well as induction of tumor progression through up-regulation of reactive oxygen species ROS and DNA damage [9,10]. On the other hand, there are solid evidences of potential antitumor effects of BITC. This compound has a

Abbreviations: ALT, alanine amine transferase; AST, aspartate amino transferase; AFP, alpha fetoprotein; BITC, benzyl isothiocyanate; c-MET, mesenchymal-epithelial transition factor; DEN, diethylnitrosamine; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethylsulfoxide; FBS, fetal bovine serum; HCC, hepatocellular carcinoma; HGF, hepatocyte growth factor; ITC, isothiocyanates; MAPK, mitogen-activated protein kinase; MMP-2, matrix metalloproteinase -2; MTT, (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide); NF-κB, nuclear factor-kappa B; pAkt, phosphorylated Akt; PBS, phosphate-buffered saline; PtdIns3K, phosphatidylinositol 3-kinases; ROS, reactive oxygen species; STAT3, signal transducer and activator of transcription 3; VEGF, vascular endothelial growth factor

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preventive activity in different types of cancers such as breast and prostate cancers induced in animal models [11,12].

Kim and his colleagues, [13] reported that BITC inhibited hepatocyte growth factor HGF induced migration of breast cancer cells and suppressed HGF mediated activation of Akt signaling. HGF and its tyrosine kinase receptor mesenchymal-epithelial transition factor c-MET pathway play a pivotal role in promoting angiogenesis, proliferation, and metastasis of HCC. This important pathway is thought to induce HCC progression through its downstream active molecules PtdIns3K/Akt, MAPK and STAT3 [14].

Limited studies have investigated the potential effect of BITC against HCC [15,16]. This study investigated the possible chemopreventive and anticancer effects of BITC on HCC experimental models as well as the possible implication of HGF /pAkt/ STAT3 axis on the proposed BITC's antitumor effects. This study introduces BITC as a potent anticancer agent; it induces apoptosis and inhibits proliferation of cancer cells. The drug induced potent inhibition of HGF and it downstream molecules pAkt and STAT3. Implication of this antineoplastic pathway was associated with decrease in angiogenic and metastatic markers. The current study presents BITC as a new agent that could be implicated in chemotherapeutic regimens directed to HCC.

2. Material and method

2.1. Drugs and chemicals

Diethylnitrosamine (DEN) and benzyl isothiocynate (BITC) were purchased from Sigma- Aldrich (St. Louis, MO, USA). HepG2 and Huh-7 cells were purchased from Vaccsera (Giza, Egypt). All other chemicals used in this research were obtained from the El-Gomhouria Company for trading Chemicals and Medical Appliances (Cairo, Egypt) and were of the highest quality/analytical grade. For invivo experiment the chemicals were prepared as follow

a 0.1 ml of diethylnitrosamine concentrated solution (1gm in 0.5 ml) was dissolved in 25 ml saline to produce a working solution (8 mg/1 ml).
b 0.2 ml of benzyl isothiocynate concentrated solution (1gm/1 ml) was dissolved 25 ml corn oil to produce a working solution (8 mg/1 ml)

2.2. In vivo experiment

2.2.1. Animals

This study was carried out on 60 male mice weighing from 20 to 30 g. Mice were obtained from the National Research Center (Dokki, Giza, Egypt) and housed in a pathogen-free facility in cages with sawdust bedding. The facilities were maintained at [25 ± 2] ˚C with 50% relative humidity and a 12-hour light: dark cycle. All mice had ad libitum access to standard rodent chow and filtered water. The study protocol and animal use were approved by The Ethical Committee of Faculty of Pharmacy, Damanhour University. The experimental procedures were done in comply with the ARRIVE guidelines in accordance with the U.K. Animals (Scientific Procedures) Act, 1986 and associated guidelines, EU Directive 2010/63/EU for animal experiments.

2.2.2. Experimental protocol

Seventy five mice were randomly allocated into five groups (15 mice /group) and were treated as follows: Group 1 (DEN group) mice were injected intraperitoneally (IP) with DEN (75 mg/kg) once a week for 4 weeks [17]. Second and third group of mice were injected with the same DEN protocol plus oral doses of BITC (20 mg/kg/day) and (10 mg/kg/day) respectively [18,12]). The fourth group of mice were used as control and treated with vehicles. The last group of mice were injected only with the high dose of BITC (20 mg/kg/day) for 10 weeks and served as drug control group (Fig. 1A).

2.2.3. Preparation of blood and tissue homogenate

At 24 h after the last dose of drug/vehicle injection, all mice were anesthetized with diethylether. Blood samples were immediately suctioned via cardiac puncture and collected into tubes then allowed to be clotted at room temperature for 60 min. The obtained blood samples were centrifuged (3000 g, 10 min, 4 ˚C). Serum of each sample was recovered and stored at - 20 ˚C until analysis.

Mice were euthanized by cervical dislocation. The liver of each animal was carefully removed. Two 0.5-cm sections of the second largest lobe were fixed in 10% formalin and further prepared for histopathological examination. The remaining portions of each liver were divided into four parts and individually stored at -80 C° until analyzed. For those latter analyses, liver homogenates were prepared in 10 vol (i.e. 0.1 g tissue in 1 ml) cold phosphate-buffered saline (PBS, pH 7.4). Each resulting mixture was centrifuged (6000 g, 20 min, 4C°). Aliquots of the derived supernatant were used for biochemical analysis and measuring protein content using a standard kit (Wokea Medical Supplies, Changchun, China).

2.3. In-vitro experiment

2.3.1. Assay of HepG2 and Huh-7 cells growth by MTT method

This study were made using HepG2 and Huh-7 cell lines. HepG2 cells are human liver carcinoma cells that express all signaling pathways related to HCC while other cell line may express only limited signaling pathways

Cytotoxicity was determined by classic Microculture tetrazolium test (MTT). The MTT assay measures the cell proliferation. In principle, the yellow tetrazolium MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) is reduced by metabolically active cells, yielding purple MTT formazan crystals which has a λ max of 540 nm and is considered to be directly proportional to the number of viable cells [19]

HCC cell lines HepG2 and Huh-7 were obtained from the Vacsera (Cairo, Egypt). Cells were plated in 96 well plate at a concentration of 5×10^3 cells/well in Dulbecco's modified Eagle's medium DMEM (Lonza Biowhittaker™, B-4800 Verviers, Belgium) supplemented with 50 µg/ml streptomycin, 50 U/ml penicillin (Lonza Biowhittaker™, B-4800 Verviers, Belgium), 2 mM glutamine, and 10% (v/v) FBS (Sigma-Aldrich, St. Louis, MO, USA) followed by incubation at 37 ˚C in 90% air/10% CO2. The microtiter plates were then incubated at 37 ˚C in 5% CO2 for 24 h to allow cell attachment. Culture medium was replaced with another 200 µl of the same medium containing different serial concentrations of BITC (1–120 µM) except on control and standard wells (Fig. 1B).

The plate was then incubated at the same conditions for 72 h. On the third day, the culture media was discarded then 10 µl MTT was added. The plates were incubated at the same conditions for 2–4 hours until the purple precipitate was visible. The supernatant was removed carefully without disturbing the formazan crystals using a multi-channel pipette. DMSO (100 µl) was added on each well for the solubilization of the formazan crystals. The microtiter plates were placed on the shaker for 3 min to ensure the homogeneity of the color. The plate was left at room temperature in the dark for 2 h. The optical density of each well was determined within 30 min using a microplate reader (Model 550, Bio-Rad, USA) set to 570 nm.

Absorbance values that are lower than the control wells indicate a reduction in the rate of cell proliferation. Conversely a higher absorbance rate indicates an increase in cell proliferation. Viability percent could be obtained by dividing average absorbance yielded in a given specified treatment concentration over the average absorbance yielded by control cells. Consequently, % inhibition could be obtained by subtracting % viability from 100. Then, IC50 was determined by extrapolating 50% viability or inhibition to its corresponding concentration.