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Bergapten inhibits liver carcinogenesis by modulating LXR/PI3K/Akt and IDOL/LDLR pathways

Shakti Prasad Pattanayak[a,*], Pritha Bose[a], Priyashree Sunita[b], Mohd Usman Mohd Siddique[c], Antonio Lapenna[d]

[a] Division of Advanced Pharmacology, Department of Pharm. Sciences & Technology, Birla Institute of Technology, Mesra, Ranchi, 835215, India
[b] Govt. Pharmacy Institute, Department of Health, Education & Family Welfare, Govt. of Jharkhand, Bariatu, Ranchi, 834009, India
[c] Division of Pharmaceutical Chemistry, Department of Pharm. Sciences & Technology, Birla Institute of Technology, Mesra, Ranchi, 835215 Jharkhand, India
[d] Academic Unit of Inflammation & Tumor Targeting, Department of Oncology and Metabolism, University of Sheffield Medical School, Beech Hill Road, Sheffield S10 2RX, United Kingdom

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ABSTRACT

Oxysterol receptors LXRs (α and β) are recently reported to be one of the novel and potential therapeutic targets in reducing cell proliferation and tumor growth in different system model. Activation of LXRs is correlated with modification of PI3K/Akt pathway. LXRs are also found to play a critical role in maintaining lipid homeostatais by regulating ABCA1, IDOL, SREBP1, LDLR and also certain lipogenic genes such as FASN and SCD1. In the present study a potential furanocoumarin, Bergapten (BeG) has been evaluated for its anticancer property on Hepatocellular Carcinoma (HCC) on LXR axis. The molecular docking analysis was carried out for BeG on LXR (α & β) using Maestro tool and compared with reference ligands. This was followed by *in vitro* (HepG2 cell lines) and *in vivo* (on NDEA induced HCC in Wistar albino rats) anticancer evaluation of BeG. The docking results revealed polar and hydrophobic interactions of BeG with LXR (α,β). The *in vitro* studies revealed the potential of BeG in lowering the accumulation of lipid droplets in HepG2 cells which was correlated with increase in LXR (α,β) protein expressions. Furthermore, the *in vivo* studies demonstrated the potential of BeG in ameliorating the cancer induced alterations in body weight, liver weight and significant restoration of the changes in mRNA and protein expressions of LXR(α,β), ABCA1, IDOL, SREBP1 and LDLR. BeG also modulated the expressions of PI3K, Akt and certain lipogenic genes like FASN and SCD1 and reduced the lipid droplets level in liver cancer cells. These results provide evidence and validates the critical role of BeG in maintaining the lipid homeostasis and justifies its anticancer potential against NDEA-induced HCC.

1. Introduction

Cancer is characterized by abnormal and uncontrolled growth of cells in any part of the body which has the ability to metastasize and invade adjacent tissues. Hepatocellular Carcinoma (HCC) ranks 5th in cancer incidence and is the 3rd leading cause of mortality worldwide [1]. About 500,000 new cases are reported annually and patients suffering from liver cirrhosis comprise 2.5% to 7% of the yearly incidence [2].

N-Nitrosodiethylamine (NDEA) present in cured and fried meals, tobacco smoke, water, cheddar cheese, various cosmetics and pharmaceutical products and even in agricultural chemicals is known to be a potent hepatocarcinogenic agent [3]. NDEA is responsible for producing reproducible liver tumors [4] as it is reported to form DNA adducts [5]. NDEA metabolism causes oxidative stress which is responsible for the cytotoxic, mutagenic and carcinogenic effect [6].

Recently, alteration of lipid metabolism is considered to be a hallmark of cancer cells [7]. Liver X receptors (LXRs) which are oxysterol receptors are encoded by genes Nhr1h3 (LXRα) and Nhr1h2 (LXRβ). These well-established ligands regulated transcription factors are associated with lipid, cholesterol, carbohydrate and other metabolism processes [8]. Recently, LXRs are recognized as anti-inflammatory transcription factors that can modulate different physiological functions [9]. Various researches suggest that LXR may serve as potential targets to prevent and treat several cancers [8,10]. The LXRs are also capable of inducing cholesterol efflux from cells that contribute to its antiproliferative effects.

LXRs activation reduces intracellular cholesterol levels by

* Corresponding author.
E-mail address: sppattanayak@bitmesra.ac.in (S.P. Pattanayak).

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Adenosine Triphosphate Binding Cassettes A1 (ABCA1) cholesterol transporters that decrease lymphocyte proliferation [11]. Increased expression of ABCA1 and Inducible Degrader of LDL receptor (IDOL) due to LXR activation triggers LDL receptor degradation. This in turn decreases cholesterol levels, thereby reducing tumor growth and survival [12]. The cytotoxic effect of LXRs is also aggravated due to cholesterol efflux mediated by ABCA1 activation [13]. Fatty acid synthase (FASN) and Acetyl co-carboxylase (ACC) have been also found in HCC as well as various other human epithelial cancer and pre-neoplastic lesions [14]. Low-density lipoprotein receptors (LDLR) are found on the cell surface and are involved in removal of low-density lipoprotein (LDL) particles by receptor mediated endocytosis from plasma. When LDLR activity is up-regulated in hepatocytes, it increases LDL clearance from circulation and thus hepatic LDLR serves as a major determining factor of cholesterol level in plasma. Myosin Regulatory Light Chain Interacting Protein (Mylip)/IDOL has been recently identified to have key regulatory role in modulating LDLR abundance [15]. In hepatic cells, LDLR abundance is decreased by Mylip/IDOL protein over expression which attenuates LDL uptake activity [16]. Mylip/IDOL protein which is excessively produced in liver due to activation of LXR signaling pathways causes reduction of LDLR protein and rise in plasma LDL. So, this serves to be a novel target in development of cholesterol lowering agents for lipid management.

Accumulation of cholesterol transporter ABCA1 induces decrease in cellular cholesterol level that reduces size of lipid rafts. This in turn, inactivates Akt pathway, lowering the cell survival ability [17]. Sterol Regulatory Element Binding Proteins (SREBPs) belonging to one of the most important transcription factor families, maintain lipid homeostatis and also regulate most enzymes involved in fatty acid synthesis [18]. SREBP1c is found to be highly expressed in liver, prostate and ovarian cancers [19]. Various studies revealed that many well-known oncoproteins tightly regulate the expression and activity of SREBP1. PI3K/Akt signaling pathway that is activated in various human cancer is also associated with regulation of cell metabolism and fatty acid synthesis [20]. It also induces *De novo* lipogenesis by enhancing expression and activity of SREBP1 [21]. Nuclear accumulation of SREBP1 is promoted by activation of constitutive Akt. SREBP is also found to activate different lipogenic genes. Study revealed that CD147 (trans-membrane glycoprotein) mediated activation of Akt, up-regulated SREBP1 and was also responsible for FASN and ACC expression, cell growth and progression of cell cycle [22].

The furanocoumarin, Bergapetn (BeG) which is the naturally occurring analogue of psoralen and isomer of methoxaselen is widely available in various plants like roots and fruits of *Angelica archangelica*L. (Apiaceae)-Angelica, Garden Angelica, European Angelica; seeds of *Apium graveolens*L. (Apiaceae)-Celery; leaves, stems and fruits of *Petroselinum crispum*(Mill.) Nym. (Apiaceae)- Parsley; Rue Oil of *Ruta graveolens*L. (Rutaceae)-Rue, Garden Rue, German Rue. *In vitro* studies reported antioxidant properties of BeG [23]. Studies also revealed, anti-inflammatory properties [24] as well as anticancer and hepatoprotective properties [25] of BeG. Various studies revealed inhibition of COX-2 [26] and topoisomerase 1 [27] by BeG. In this study, we designed to investigate the potential of BeG in maintaining lipid homeostatis in NDEA-induced liver carcinogenesis in Wistar albino rats and tried to evaluate its' underlying molecular mechanisms.

2. Materials and methods

2.1. Molecular docking & human LXR reporter assay

The Schrödinger, Maestro 8.5 was used for molecular modeling studies, Chem Draw 10.0 program was used to convert the chemical structures into 3D conformation. Ligand preparation was done using lig-prep module (Schrodinger, Maestro 8.5). The X-ray crystal structures of LXRα which is a dimer (chain A, B) having tert-butyl benzoate analogue (4KQ) as co-crystalized ligand (PDB entry: 5AVL), LXRβ (a tetramer with chain A, B, C, D) having 25-epoxycholesterol (PDB entry: 1P8D with simvastatin (PDB entry: 1HW9) as their co-crystallized ligands respectively and Akt (PDB ID: 5KCV), PXR (PDB ID: 3EY4) and FXR (PDB ID: 4OIV) were downloaded from RCSB protein database (http://www.rcsb.org). Chain A in LXR (α,β) was used in the docking procedure. After the software validation by re-docking method, BeG was docked into the active site of the LXRα, LXRβ within the radius of 6.5A° (default). Maestro software is based on the constructive incremental algorithm. Other parameters were set referring the default values. The docking scores and suitable binding patterns were reported comparing with the reference ligands.

The reporter study was performed using the Human LXR (α&β) Assay Panel Kit with manufacturer's instructions (Indigo Biosciences, PA, USA). Briefly after thawing of compound screening medium (CSM) in 37 °C water bath, CSM was used to prepare the appropriate dilutions of BeG (2x concentration). For preparing positive control, a 10 mM stock solution of TO901317, (a potent reference agonist for both LXRα and LXRβ) was used following 7-point treatment series, with concentrations presented in 3-fold decrements, provides a suitable dose-response: 6000, 2000, 667, 222, 74.1, 24.7, 8.23 nM, and including a 'no treatment' control. Then the reporter cells were rapidly thawed by transferring a 5.0 ml volume of 37 °C cell recovery medium into the tube of frozen cells. The tube of reporter cells was recapped and immediately placed it in a 37 °C water bath for 5–10 min so that the resulting volume of cell suspension be 6.0 ml. Following this, four sterile 8-well strips were mounted into the blank assay plate frame and the tube of reporter cell Suspension is retrieved from the water bath. Then for the agonist-mode assays, the tube of LXR reporter cells were inverted gently several times to disperse cell aggregates and gain a homogenous cell suspension. To this 100 µl of cell suspension was dispensed. Then 100 µl per well of 2x-concentration treatment media was dispensed into appropriate wells of the assay plate and the assay plate was transferred into a 37 °C, humidified 5% CO_2 incubator for 22–24 h. Next day after the tubes of detection substrate and detection buffer were equilibrated to room temperature and each tube was gently inverted several times to ensure homogenous solutions. Media contents from each well was removed after 22–24 h of incubation. Then, 100 µl of luciferase detection reagent was added to each assay well plate and was allowed to rest at room temperature for at least 5 min before luminescence quantification (Molecular Devises Luminometer equipment -SpectraMax i3x, Molecular Devices, CA, USA).

2.2. Cell culture [28]

The HepG2 cells were maintained in continuous culture at 37 °C temperature in Dulbecco's modification of Eagle's medium (DMEM) supplemented with 10% FBS, 2 mM L-glutamine, 100 units/ ml penicillin, 100 µg/ml streptomycin, 0.5 µg/ml fungizone. When required for assays confluent monolayer of HepG2 cells was washed twice with sterile PBS. Thereafter, Trypsin-EDTA solution (250 mg Trypsin, 30 mg EDTA in 100 ml PBS) was added to de-adhere the cells from the culture flask. Cells were de-adhered, collected and centrifuged at 200 × g for 5 min. Cultured HepG2 cells were treated with different concentrations of BeG (10, 20 and 50 mM) for 12, 24, 48 h. BeG was dissolved in DMSO, and the final concentration of the vehicle was < 0.1%. DMSO (solvent) has been added to the control regimen (0 mM of BeG). Immunofluorescence analysis of the lipid droplets was carried out as discussed below under section immune-fluorescence analysis. Also, the protein and mRNA analysis of LXRα and LXRβ were carried out using western blot and RT-qPCR techniques (discussed below).

2.3. Experimental animals

For this study 50–55 days old Wistar albino rats were used and they were acquired from National Institute of Nutrition, Hyderabad. Animals were housed in polystyrene, well aerated cages at normal atmospheric