Chemico-Biological Interactions xxx (2015) xxx–xxx

Contents lists available at ScienceDirect

Chemico-Biological Interactions

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

In vitro and *in vivo* evaluation of novel cinnamyl sulfonamide hydroxamate derivative against colon adenocarcinoma

Neetinkumar D. Reddy [a], M.H. Shoja [a], B.S. Jayashree [b], Pawan G. Nayak [a], Nitesh Kumar [a], V. Ganga Prasad [a], K. Sreedhara R. Pai [a], C. Mallikarjuna Rao [a,*]

[a] Department of Pharmacology, Manipal College of Pharmaceutical Sciences, Manipal University, Manipal 576104, Karnataka, India
[b] Department of Pharmaceutical Chemistry, Manipal College of Pharmaceutical Sciences, Manipal University, Manipal 576104, Karnataka, India

ARTICLE INFO

Article history:
Received 26 August 2014
Received in revised form 3 February 2015
Accepted 18 March 2015
Available online xxxx

Keywords:
Colon cancer
Cinnamic acid
HDAC
Apoptosis
Caspase 3/7
1,2-Dimethyl hydrazine (DMH)

ABSTRACT

The potential of cinnamic acid as an anti-inflammatory and anti-cancer agent has been studied previously. In our investigation, novel bio-isosters of cinnamyl sulfonamide hydroxamate were synthesized, characterized and confirmed for their structure and evaluated for cytotoxicity. Three NCEs namely, NMJ-1, -2 and -3 showed cell-growth inhibition in 6 human cancer cell lines with IC_{50} at the range of 3.3 ± 0.15–44.9 ± 2.6 μM. The hydroxamate derivatives of cinnamyl sulfonamide are reported inhibitors of HDAC enzyme. Thus, the effectiveness of these molecules was determined by whole cell HDAC assay in HCT 116 cell line. NMJ-2 (0.41 ± 0.01 μM) exhibited better enzyme inhibition (IC_{50}) compared to SAHA (2.63 ± 0.07). In order to evaluate induction of apoptosis by treatment, Hoechst 33342 and AO/EB nuclear staining methods were used. Further, cell cycle analysis, Annexin V binding and caspase 3/7 activation assays were performed by flow cytometry where NMJ-2 significantly arrested the cell cycle at G_2/M phase, increased Annexin V binding to the cell surface and activation of caspase-3/7. Bax/Bcl-2 ratio was observed by Western blot and showed an increase with NMJ-2 treatment. This was comparable to standard SAHA. The acute toxicity study (OECD-425) showed that NMJ-2 was safe up to 2000 mg/kg in rats. 1,2-Dimethyl hydrazine (DMH) was used to produce experimental colon adenocarcinoma in Wistar rats. 5-FU and NMJ-2 (100 mg/kg *p.o.* and 10 mg/kg *i.p.* once daily for 21 days, respectively) were administered to the respective groups. Both treatments significantly reduced ACFs, adenocarcinoma count, TNF-α, IL-6, nitrite and nitrate levels in colonic tissue. Our findings indicate that NMJ-2 has potent anti-cancer activity against colon cancer, by acting through HDAC enzyme inhibition and activation of intrinsic mitochondrial apoptotic pathway, with additional anti-inflammatory activity.

1. Introduction

Cancer has reached epidemic proportions globally and accounts for 12 million cases worldwide and 7.9 million deaths [1]. It accounts for 13% of all deaths each year with the most common being lung, stomach, colorectal, liver and breast cancer [2,3]. It is found that generally cancer risk rises with old age [4]. Colon cancer is now a common malignancy in various parts of the world. Numerous cell-signaling pathways have pivotal roles to play in the proliferation of malignant cells. The search for newer avenues in the treatment of cancer has led research into the elucidation of signaling pathways. The complexity associated with this disease has led scientists to identify targets for the different phases of tumor formation via cell differentiation, proliferation, cell cycle

Abbreviations: ACF, aberrant crypt foci; AO/EB, acridine orange/ethidium bromide; CPCSEA, Committee for the Purpose of Control and Supervision of Experiments on Animals; CMC, carboxymethylcellulose; DMEM, Dulbecco's minimum essential media; DMSO, dimethyl sulfoxide; DMH, 1,2-dimethyl hydrazine; FBS, fetal bovine serum; 5-FU, 5-fluorouracil; HDAC, histone deacetylase; HDACi, HDAC inhibitors; HepG2, human liver adenocarcinoma; HCT 116, human colon adenocarcinoma; IL-6, interleukin-6; *i.p.*, intra-peritoneal; MCF-7, human breast (ER+) adenocarcinoma; MDA-MB-231, human breast (ER−) adenocarcinoma; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NCE, new chemical entity; NO, nitric oxide; OECD, Organization for Economic Co-operation and Development; PC3, human prostate adenocarcinoma; PBS, phosphate buffer saline; PI, propidium iodide; *p.o.*, per oral; S.E.M., standard error of mean; SAHA, suberoyl anilide hydroxamic acid; SH-SY5Y, human neuroblastoma; TNF-α, tumor necrosis factor-α; Vero, African green monkey kidney epithelial cell line.

* Corresponding author. Tel.: +91 0820 2922482 (O).
E-mail addresses: mallikin123@gmail.com, mallik.rao@manipal.edu (C. Mallikarjuna Rao).

http://dx.doi.org/10.1016/j.cbi.2015.03.015
0009-2797/

check point defect, migration and cell survival through onco-gene activation etc. [5].

Cancer is caused by abnormal epigenetic modifications in addition to multiple genetic mutations. Certain validated targets such as tyrosine kinase, farnesyl transferase, histone deacetylase (HDAC), aromatase, etc. play a central role in the development of target specific anticancer agents. Histone deacetylase (HDAC) enzyme overexpression, linked to many cancer types, is responsible for tumor suppressor gene silencing and activation of proto-oncogenes. The altered expression of HDACs play a direct or indirect role in tumor development [6]. HDAC inhibitors (HDACi), a new class of anti-cancer agents, play a crucial role in epigenetic modification, activation of tumor suppressor genes, cell cycle regulation and apoptosis induction in cancer cells. Several HDACi are under pre-clinical or clinical trials. The limitations of these molecules include thrombocytopenia, cardiac problems and poor efficacy against solid tumors etc. [7]. Thus, there is a scope to improve their safety and efficacy.

From time immemorial, use of Tolu balsam as an anti-inflammatory and anti-cancer agent has been well documented owing to the presence of esters of benzoic and cinnamic acid [8]. In recent studies, cinnamic acid derivatives showed anti-cancer activity via HDAC enzyme inhibition [9]. Based on these findings, we made modifications in the cinnamic acid moiety to obtain cinnamyl sulfonamide hydroxamate derivatives. The design evolved around the concept of bio-isosterism and was used as a tool in lead modification [10]; where, the benzene ring of the known available HDACi such as SAHA/PDX101, was conveniently replaced by electronically equivalent ring counterpart such as thiophene. The biological activities of these newly formed NCEs were compared with their phenyl counterparts. Hence, the aim of the present study was to synthesize novel cinnamyl sulfonamide-hydroxamate derivatives and evaluate them for anti-cancer efficacy by *in vitro* and *in vivo* colon adenocarcinoma models.

2. Materials and methods

2.1. Common chemicals and reagents

Starting materials of synthetic grade were obtained from (Sigma–Aldrich Co. LLC, St. Louis, MO, USA; Merck KGaA, Darmstadt, Germany; Spectrochem Pvt. Ltd., Mumbai, MH, India; TCI Co. Ltd., Tokyo, Japan). Dulbecco's modified Eagle's medium (DMEM), phosphatase inhibitor cocktail, protease inhibitor cocktail, propidium iodide (# P4170), SAHA (# SML0061), Boc-Lys(Ac)-AMC substrate (# SCP0168) and Nonidet-P 40 (NP-40), Griess' reagent (# 03553), vanadium (III) chloride (# 208272) were obtained from Sigma–Aldrich Co. LLC, MO, USA; fetal bovine serum (FBS) (Gibco; # 10437036) was obtained from Invitrogen BioServices India Pvt. Ltd., Bangalore, KA, India; 5-fluorouracil (5-FU) was procured from Biochem Pharmaceutical Industries Ltd., Mumbai, MH, India. All chemicals and buffers for Western blotting were obtained from Bio-Rad Laboratories Inc., Hercules, CA, USA. Bax (# 2772S), Bcl-2 (# 2876S), GAPDH (# 2118S), Anti-rabbit IgG HRP-linked (# 7074S) antibodies were procured from Cell Signaling Technology Inc., Danvers, MA, USA. Tissue culture plastic wares and materials were purchased from Tarsons Products Pvt. Ltd., Bangalore, KA, India; Muse™ Annexin V & Dead Cell Kit (Cat# MCH100105), Muse™ Caspase-3/7 Kit (# MCH100108) (Merck KGaA, Darmstadt, Germany); 1,2-dimethylhydrazine hydrochloride [DMH] (# D0742) from TCI Co. Ltd., Tokyo, Japan; IL-6 ELISA kit (# KRC3011), TNF-α ELISA kit (# KRC3011) was purchased from Invitrogen BioServices India Pvt. Ltd., Bangalore, KA, India. All other reagents, chemicals and solvents used in study were of analytical grade quality.

2.2. Equipment for synthesis and characterization of synthesized compounds

Melting point of the synthesized test compounds were determined using capillary melting point apparatus (Toshniwal Systems and Instruments Pvt. Ltd., Chennai, TN, India). The reaction status was checked by TLC on pre-coated silica gel plates (Merck # 60F254). Spots were visualized under both long and short UV range using UV lamp (366 or 254 nm) and iodine chamber. The R_f values for the synthesized test compounds were determined using chloroform: methanol (9:1) solvent system. Further, the test compounds were purified by silica gel column chromatography. The IR spectra were recorded using IR spectrometer (Model FTIR-8300, Shimadzu Co., Kyoto, Japan) using KBr pellets. ^1H and ^{13}C NMR were recorded at 400 MHz (Model Ascend 400, Bruker Biosciences Corporation, Billerica, MA, USA) using DMSO (D6) as solvent. Mass spectra were recorded using LC-MS (ESI) (Model LCMS-2010A, Shimadzu Co., Kyoto, Japan). CHN-S elementary analyses were done by Vario EL Ver III CHNS analyzer from Elemental Analysensysteme, GmbH, Germany. NMR, Mass spectra and CHN-S analysis fully supported the final structures of the test compounds. Purity of the test compounds was established on RP-HPLC unit (Shimadzu Co., Kyoto, Japan) with a PDA detector (254 nm) using a Hichrom C18 (250 × 4.6 mm i.d., 5 μm) column with acetonitrile as solvent in pump A and aqueous solution of 0.1% formic acid (pH 6.0) as solvent in pump B by gradient elution. Flow rate of 1.0 ml/min was maintained with a run time of 35 min and column temperature of 30 °C.

2.3. Cell culture and maintenance

All the cell lines (HCT 116, MCF-7, MDA-MB-231, HepG2, SH-SY5Y and Vero) were procured from the National Centre for Cell Science, Pune, MH, India. The cells were maintained in high glucose DMEM medium with 10% FBS and 1% penicillin–streptomycin, at 37 °C in a CO_2 incubator (NU-5510E, NuAire Inc., Plymouth, MN, USA). Trypan blue dye exclusion method was used to check viability of cells and >95% viable cells in culture were used through the experiments. The MTT cell viability assay was performed in all 6 cancer cell lines and rest of the *in vitro* studies were done in HCT 116 colon cancer cell line. Three independent experiments in triplicates were done for the all *in vitro* procedures ($n = 3$).

2.4. Animals, dose administration and treatments

Wistar rats (120–150 g) were used in the study from well-maintained in-house bred, of the Central Animal Research Facility, Manipal University, Manipal, Karnataka, India. All animal experiments were conducted according CPCSEA guidelines, Government of India and after obtaining the experimental protocol approval (No. IAEC/KMC/19/2012) from the Institutional Animal Ethics Committee (Animal Use and Care Committee). Animals were acclimatized in polypropylene cages in experimental room and given standard food pellet rodent diet and water *ad libitum* and kept under controlled humidity conditions 45–55%, temperature 25 ± 2 °C, ventilation 10–12 exchanges/h and 12:12 h light and dark cycle. 5-FU was used as standard drug at a dose of 10 mg/kg, *i.p.* injection which is one twenty fifth (1/25th) of human dose, converted to the rat dose (Paget and Barnes, 1964). NMJ-2 dose was selected based on acute toxicity study limit test (up to 2000 mg/kg). One twentieth (1/20th) of the maximum tested safe dose, i.e. 100 mg/kg was selected for anti-cancer study and administered for a period of 21 days. All other test compounds were prepared as suspensions in 0.25% carboxymethylcellulose (CMC) and administered through oral route.

Please cite this article in press as: N.D. Reddy et al., *In vitro* and *in vivo* evaluation of novel cinnamyl sulfonamide hydroxamate derivative against colon adenocarcinoma, Chemico-Biological Interactions (2015), http://dx.doi.org/10.1016/j.cbi.2015.03.015