European Journal of Pharmacology 764 (2015) 508–519

Contents lists available at ScienceDirect

European Journal of Pharmacology

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

Molecular and cellular pharmacology

Phosphodiesterase inhibitor, pentoxifylline enhances anticancer activity of histone deacetylase inhibitor, MS-275 in human breast cancer *in vitro* and *in vivo*

Saranya Nidhyanandan [a,c,*], Thippeswamy S. Boreddy [b], Kothapalli B. Chandrasekhar [c], Neetinkumar D. Reddy [a], Nagaraj M. Kulkarni [a], Shridhar Narayanan [d]

[a] Department of Biology, Drug Discovery Research, Orchid Chemicals and Pharmaceuticals Ltd., Old Mahabalipuram Road, Sozhanganallur, Chennai 600119, Tamil Nadu, India
[b] Department of Biomedical Science, College of Pharmacy, Shaqra University, Al-Dawadmi, Kingdom of Saudi Arabia
[c] Jawaharlal Nehru Technological University Anantapur, Anantapur, 515 002 Andhra Pradesh, India
[d] Foundation for Neglected Disease Research, Sir M Visvesvaraya Institute of Technology, International Airport Road, Yelahanka, Bangaluru 562157, India

ARTICLE INFO

Article history:
Received 2 June 2015
Received in revised form
17 July 2015
Accepted 20 July 2015
Available online 21 July 2015

Keywords:
HDAC
MS-275
PDE
Pentoxifylline
Apoptosis
Xenograft

ABSTRACT

MS-275, a histone deacetylase inhibitor (HDACi), is undergoing clinical trials for treatment of various cancers. Pentoxifylline, a nonselective phosphodiesterase (PDE) inhibitor, has been shown to increase the effectiveness of antitumor chemotherapy. In the present study, the potential anti-cancer activity of MS-275 in combination with pentoxifylline in panel of cell lines and human breast cancer xenograft model were examined.

A Panel of cancer cell lines were treated with MS-275 and pentoxifylline to determine their impact on cellular proliferation, cell cycle regulation, apoptosis, anti-angiogenesis. The *in vivo* activities of MS-275 and pentoxifylline were assessed in a Matrigel plug angiogenesis model and human breast cancer (MDA-MB-231) xenograft model.

Combination of MS-275 with pentoxifylline showed enhanced anti-proliferative activity in a panel of cancer cell lines (HCT 116, MCF-7, PC3 and MDA-MB-231). Apoptotic studies performed using, Hoechst staining and cell cycle analysis reveal that this combination at the lower concentrations induces apoptosis downstream of the HDAC inhibition and PDE regulation. Further, combination showed enhanced antiangiogenic activity in Matrigel tube formation assay using HUVECs and in Matrigel plug assay *in vivo*. A significant inhibition ($P < 0.001$) of tumor growth was observed in mice bearing MDA-MB-231 breast cancer xenograft treated with the combination of MS-275 (5 mg/kg p.o.) and pentoxifylline (60 mg/kg i.p.) than treatments alone, without much signs of toxicity.

Taken together, our study demonstrated enhanced anticancer activity of MS-275 and pentoxifylline combination both *in vitro* and *in vivo* with reduced toxicity. However, further studies are required to understand the mechanism for this combination effect.

1. Introduction

Chemotherapy drugs are most effective when given in combination. The fundamental rationale for combination therapy in the treatment of cancer is to use approaches that work by different mechanisms of action thereby targeting different key signal transduction pathways resulting in destruction of cancer cells more efficiently and in eluding the cellular resistance mechanisms (Li et al., 2012). Besides this, the combination may in fact give additive or even synergistic effects, so that a reduction of the dose of the most toxic component is sometimes feasible.

One of the promising approach for cancer therapy is modulation of the intracellular cyclic nucleotides, cyclic adenosine monophosphate (cAMP) or cyclic guanosine monophosphate (cGMP) (McEwan et al., 2007). The impairment of cAMP and cGMP generation by regulation of phosphodiesterases (PDEs) has been shown in various cancer pathologies viz. multiple human carcinomas including metastatic breast cancers, colon adenocarcinoma, bladder squamous carcinoma, prostrate and lung cancers (Kraft et al., 2006). Pentoxifylline, (a methyl xanthine derivative) regulates PDE non-specifically, and used for the treatment of

* Corresponding author at: Department of Biology, Drug Discovery Research, Orchid Chemicals and Pharmaceuticals Ltd., Old Mahabalipuram Road, Sozhanganallur, Chennai 600119, Tamil Nadu, India.
E-mail address: Saran.nithy@gmail.com (S. Nidhyanandan).

http://dx.doi.org/10.1016/j.ejphar.2015.07.048
0014-2999/

intermittent claudication or other circulatory disorders. Pentoxifylline possess anti-tumour and anti-metastatic activity in combination treatment (Bravo-Cuellar et al., 2010; Barancik et al., 2012). Hence, agents that raise levels of cAMP or cGMP by inhibiting their breakdown by PDEs have gained prominence as potential targets for anticancer therapy (Murata et al., 1999; Hirsh et al., 2004). However, the studies have shown limited efficacy of PDE inhibitors in cancer therapy when used alone. Therefore combination of PDE inhibitors with chemotherapeutic agents may be a rational approach for the treatment of cancer (Goel and Gude, 2014). MS-275 is a class I selective histone deacetylases (HDACs) enzyme inhibitor, currently in clinical trials. HDAC enzyme is involved majorly in regulating the acetylation of a variety of histone and non-histone proteins (Khan et al., 2008). HDACs are also having a role in controlling the transcription and regulation of genes involved in cell cycle control, proliferation, survival, DNA repair and differentiation (Krusche and Wulfing, 2005). HDAC inhibitors as a standalone therapy has limited clinical benefit for patients with solid tumors because of its reported toxicity and frequently altered HDAC expression in hematologic and solid tumor malignancies. Most preclinical studies, particularly in solid malignancies, have observed a cytostatic response to HDAC inhibitors when used as monotherapies, but when combined with radiation, chemotherapy, or other targeted agents, these drugs produce a more powerful cytotoxic response (Postiglione et al., 2011; Kato et al., 2007).

The findings on the variant expression of PDE and HDAC enzymes in different tumors offer promising insight for the selective inhibition of these enzymes for combination therapy in cancer (Wallis et al., 1999). Further, when PDE inhibitor used in combination with HDAC inhibitor may allow lower dosage of these agents, resulting into reduced side effects and increased anticancer activity. Taking a step further in this direction, in our present study, we have evaluated the anti-tumor activity of PDE inhibitor (pentoxifylline) in combination with HDAC inhibitor (MS-275) both *in vitro* and *in vivo*.

2. Materials and methods

2.1. Chemicals

MS-275 and pentoxifylline were synthesized at Orchid Chemicals and Pharmaceuticals Ltd., Chennai, India. Chemicals of reagent grade (for chemical synthesis) or Cell/Molecular biology grade (for cell culture work) were obtained from standard sources.

2.2. Cell culture

MDA-MB-231, MCF7, HCT-116 and PC3 cell lines purchased from ATCC were cultured in Dulbecco's Modified Eagle Medium (DMEM) with 10% heat-inactivated fetal bovine serum (FBS, GIBCO), 100-units/ml penicillin and 100-μg/ml streptomycin (GIBCO). Cells were cultured at 37 °C in a humidified 5% CO_2 atmosphere and were passaged twice weekly. HUVEC cell line was purchased from LONZA and cultured in Endothelial Basal Medium (EBM). Cells were cultured at 37 °C in a humidified 5% CO2 atmosphere and were passaged when 70% confluent.

2.3. Cell viability

Panel of cells were seeded in 96-well culture plates at a density of 5×10^3 cells per well and were allowed for overnight adherence (Vichai and Kirtikara, 2006). Then the cells were incubated with different concentrations of both PDE and HDAC inhibitor alone and in combination (the doses of the individual drug utilized were chosen based on the result of dose response curves) and incubated for 48 h at 37 °C in CO_2 incubator. To each well of culture plate 30% of ice cold TCA was added (10% to the well) and incubated at 4 °C for 1 h. Following the incubation the culture plates were then washed for four times in slow running tap water and then 0.057% (wt/vol in 1% acetic acid) sulforhodamine B (SRB) solution was added to each well for incubation at room temperature for 30 min. The plates were quickly rinsed for four times with 1% acetic acid to remove unbound dye. The plates were then allowed to dry in room temperature and the protein bound dye was solubilized by adding 10 mM tris base and measured the OD at 530 nm spectrophotometrically. Combination index (CI) for drug interaction was calculated using the CompuSyn software (ComboSyn, Inc.; Paramus, NJ).

2.4. Flow cytometric cell cycle analysis

MDA-MB-231 Human breast cancer cells were seeded in 6-well culture plates at a density of 1×10^6 cells per well and after overnight adherence, incubated with the indicated concentration of test compounds for 48 h. The cells were detached by trypsinisation and mixed with floating cells, centrifuged and washed with PBS. The cell pellets were fixed in 70% ice-cold ethanol and stored at −20 °C. The cells were washed with PBS before nuclear staining with propidium iodide (Sigma-Aldrich) in 1% Triton X-100 and RNase A (Sigma). The stained cells were analyzed using Beckman Coulter Gallios Flow. Cell-cycle profiles were analyzed using FlowJo version 10 (Tree Star) (Chen et al., 2011).

2.5. Detection of apoptosis

For analyzing chromatin condensation with Hoechst staining (Jiang et al., 2010), MDA-MB-231 cells were seeded on 24 well plates (10×10^3 cells/ml), treated with different concentrations of test and standard for different time points. The cells were fixed with 4% Para formaldehyde (Sigma #158127) and stained with Hoechst 33258 stain (Sigma #H6024) for 10 min and viewed under UV filter sets with LEICA DMIRB microscope. Nuclear condensation was identified by an increase in the fluorescence compared to the healthy nucleus and the DNA fragmentation was identified by the appearance of micronuclei. The percentage of cells with apoptotic characteristics was scored in percentage against the total number of cells in the representative area. Counting approximately 100 to 150 cells from three different areas in three different wells did the quantification. A standard graph was plotted for percentage of chromatin condensation against the concentration of drug used. The statistical values are expressed as the mean (+/− S.E.M) from at least three different wells.

2.6. Western blot analysis

Whole-cell extract was generated from cell pellets in mammalian protein extraction buffer (Organic buffer + 10 mM NaCl + Detergent pH = 7.5 #28-9412-79 GE healthcare Bio-sciences corp.) supplemented with 1X-Protease inhibitor cocktail (Calbiochem Cat #539131), 1X Phosphatase inhibitor cocktail (Calbiochem Cat #524625). Protein content was determined using BCA (Pierce) and samples were prepared in SDS, bromophenol blue, and 100 mg/mL DTT, and boiled for 5 min at 99 °C. The total protein was resolved on a 12.5% SDS-PAGE and blotted on to a nitrocellulose membrane and the blots were then probed with antibodies against cleaved caspase 3, 8, 9 (Cell signaling) and PARP (Full-length and cleaved PARP) (Santa Cruz SC-7148), Acetyl H3 histone (Upstate #06-599) β-actin (Sigma #A1978). Horseradish peroxidase–conjugated secondary antibodies were used (Maggio et al., 2004), ECL (Enhanced chemiluminescence; Pierce) for