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A 35 kDa *Phyllanthus niruri* protein suppresses indomethacin mediated hepatic impairments: Its role in Hsp70, HO-1, JNKs and Ca^{2+} dependent inflammatory pathways

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ABSTRACT

The present study has been conducted to explore a novel strategy to modulate the unfavourable effects of indomethacin by *Phyllanthus niruri* protein (PNP) and the underlying mechanism PNP exploits for the amelioration of that pathophysiology. In hepatocytes, indomethacin enhanced reactive oxygen species (ROS), reduced intracellular antioxidant capacity, up regulated mitogen activated protein kinase (MAPKs), disrupted mitochondrial membrane potential, activated apoptotic pathways and there by reduced the viability of the hepatocytes. Simultaneous treatment with PNP modulated these detrimental actions of the drug and retained cell viability. Similarly, in mice, indomethacin elevated serum marker enzymes (e.g. Alanine Transaminase), decreased antioxidant enzyme activities, elevated oxidations of lipids and proteins, increased intracellular calcium overload mediated endoplasmic reticular stress (ER stress) pathways, up regulated the pro-inflammatory cytokines and there by leading to the mitochondrial dependent caspase-3 activation and poly-ADP ribose polymerase (PARP) cleavage. Moreover investigation of these inherent molecular pathways exhibited that these alterations are associated with up regulation of MAPKs, inducible nitric oxide synthase (iNOS), heme oxygenase-1 and down regulation of survival proteins. However, PNP suppressed those apoptotic indices as evidenced from histopathological studies and DNA fragmentation analysis. Combining, results suggest that PNP could possibly provide a protection against indomethacin-induced hepatic pathophysiology.

1. Introduction

The Non-steroidal anti-inflammatory drugs (NSAIDs) are clinically prescribed mostly for anti-inflammatory purposes (Pal et al., 2010; Wolfe et al., 1999), because of their cyclooxygenase (COX) inhibition property (Vane, 1971). With the advancement of clinical research along with diagnostic procedures, it has been thoroughly recognized that acute or chronic treatment with NSAIDs (like aspirin, indomethacin, etc.) induces severe gastro-intestinal lesions, splenic erosions and hepatic dysfunctions (Abdel-Raheem, 2010; Asano et al., 2009; Bhattacharyya et al., 2014; Nakajima et al., 2012; Sinha et al., 2007, 2015). Emerging evidences from pharmacokinetics and pharmacodynamics studies proposed that indomethacin (Ind), could interact with the complex I of mitochondrial electron transport chain to inactivate aconitase (Chatterjee et al., 2006; Sinha et al., 2013, 2015). Once inactivated, aconitase is capable of releasing iron from the [4Fe-4S] cluster, resulting in disruption of iron metabolism and promote oxidative stress by producing hydroxyl radical (Lanas et al., 2005; Pal et al., 2010; Wallace, 2000). Available literatures provide a consensus that i) inhibition of the cyclooxygenases (COXs); ii) NSAID-induced ROS-mediated apoptosis and iii) production of •OH from free iron via Fenton chemistry which are collectively responsible for the

Abbreviations: ALT, Alanine Transaminase; BSA, bovine serum albumin; CAT, catalase; DCFDA, 2,7-dichlorofluorescein diacetate; FACS, Fluorescence activated cell sorter; FBS, Fetal bovine serum; FRAP, Ferric Reducing/Antioxidant Power; GSH, glutathione; GSSG, glutathione disulfide; GST, glutathione S-transferase; GR, glutathione reductase; HO-1, heme oxygenase-1; HSP-70, Heat Shock Protein 70; MDA, malonaldehyde; MAPK, mitogen-activated protein kinases; NF-κB, nuclear factor kappa B; Nrf-2, Nuclear factor (erythroid-derived 2)-like 2; NSAIDs, Non-Steroidal Anti Inflammatory Drugs; PARP, Poly ADP Ribose Polymerase; PNP, Phyllanthus niruri protein; ROS, reactive oxygen species; SOD, superoxide dismutase.

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pathogenesis of NSAIDs induced pathophysiology (Asano et al., 2009; Bhattacharyya et al., 2014; Sinha et al., 2015).

Despite substantial efforts in the past, the protective agent to combat the deleterious action of indomethacin remains a subject of exploration. Phytomedicinal therapy could be a probable anti-dote to trump over this toxic effects. In this connection, herbal bioactive compounds can aid as complementary and alternative medicine in the clinical prospect. Recently, researchers from our laboratory established that morin showed its mitigating efficacy against indomethacin induced gastropathy (Sinha et al., 2015). Now, we are interested in a 35 kDa herbal antioxidant protein molecule isolated and purified from *Phyllanthus niruri* as therapeutic agents. Furthermore, our previous studies with this protein (PNP) demonstrated the alleviation affectivity against iron induced cytotoxicity in mouse primary hepatocytes (Bhattacharyya et al., 2013) as well as NSAIDs (aspirin) mediated hepatic impairments in animal model (Bhattacharyya et al., 2014) through its antioxidant as well as cytoprotective property. *Phyllanthus niruri*, (family Euphorbiaceae) has been utilized as folklore medicine since many decades. In connection with that, two active ingredients, phyllanthin (Harish and Shivanandappa, 2006) and corilagin (Cheng et al., 1995) were isolated and well characterized from the organic extracts of this herb. The investigators explored that not only the aqueous extract but also the isolated protein from the herb retain antioxidant property (Bhattacharjee and Sil, 2007; Chatterjee and Sil, 2006; Sarkar and Sil, 2007). Ultimately, a 35 kDa novel antioxidant protein molecule (PNP) was purified from this herb to homogeneity. Moreover, the MS-MS analysis showed that this protein possesses unique nature in the NCBI non-redundant databases (Sarkar et al., 2009).

In the field of therapeutic research major focus has generally been paid to indomethacin mediated gastropathy than non-targeted impairments on hepatic tissues, as they are very less symptomatic and tough to diagnose. We, therefore, designed our present study to reveal whether PNP could alleviate indomethacin-mediated hepatotoxicity and if so, what signalling alleyways it employs for its amelioration in both *in vitro* along with *in vivo* system. The outcome of the overall study might explore the beneficial efficacy of PNP as an eco-friendly outstanding promising template for future drug discovery and development.

2. Materials and methods

2.1. Plant

Phyllanthus niruri, obtained from Bose Institute experimental farm, is a herb belonging to the family Euphorbiaceae.

2.2. Animals

Eight weeks old male Swiss albino mice weighing approximately 22–25 g were acclimatized under laboratory environments for 2 weeks before beginning the experiment. The animals were maintained on the standard diet, under standard conditions of temperature and humidity. These animals were used for experimental purpose by following the guidelines of the Institutional Animal Ethical Committee (IAEC), Bose Institute, Kolkata (the permit number is IAEC/BI/3(I) cert./2010), CPCSEA (Committee for the Purpose of Control & Supervision on Experiments on Animals), Ministry of Environment & Forests, New Delhi, India (1796/PO/Ere/S/14/CPCSEA) and IAEC.

2.3. Chemicals and other reagents

Indomethacin, BSA and Bradford reagent were purchased from Sigma-Aldrich Chemical Company, (St. Louis) USA. Kits for ALT measurement were purchased from Span diagnostic Ltd., India. All the other chemicals, tris buffer, vitamin C were purchased from Sisco research laboratory (Mumbai, India). All antibodies were purchased from Cell Signaling Technology and abcam (Cambridge, UK).

2.4. Isolation of protein from Phyllanthus niruri

The protein has been isolated from the fresh leaves of the plant *Phyllanthus niruri*, following the procedure of Sarkar et al., (2009). Briefly, all the fresh young leaves of the plant were homogenized in 50 mM phosphate buffer (pH 7.4), followed by centrifugation was carried out at 15,000 g and the soup was brought to 60% ammonium sulphate saturation. After centrifugation the pellet was dialysed against 50 mM phosphate buffer. It was applied to a DEAE cellulose column and the column was eluted in the same buffer using a linear gradient of 0–1 M NaCl. Two major peaks were observed and the protein fractions having maximum biological activity (active peak content) were subjected to gel filtration chromatography and re-chromatography using a gel filtration column [BIOSEP-SEC-S200, 600 × 7.8 mm] attached to the HPLC. The material of the active peak was subjected to rechromatography under identical conditions and the protein of the active fractions was utilized for the subsequent experiments. The homogeneity and the molecular weight of the protein were checked by SDS-PAGE with known molecular weight marker proteins (25–225 kDa). At every step of protein purification process the biological activity of the isolated protein has been detected as described elsewhere (Bhattacharyya et al., 2013).

2.5. Isolation of hepatocyte from mouse liver

Hepatocytes were isolated from the liver of the experimental animals upon collagenase treatment and finally suspended in DMEM (with 10% FBS)(Sarkar and Sil, 2006). The suspension was accustomed to attain ~1 × 10^6 cells/ml.

2.6. Evaluation of optimum dose and time dependent activity of indomethacin

To evaluate the optimum dose of indomethacin (Ind), hepatocytes (containing 1 ml cell suspension ~1 × 10^6 in each) were incubated with six different doses (25, 50, 100, 200, 300 and 400 μM) of Ind at 37 °C temperature and MTT assay was performed(Ghosh et al., 2011b).

For the time-dependent effect of Ind, hepatocytes (containing 1 ml cell suspension ~1 × 10^6 in each) were incubated with Ind at a particular dose (200 μM) for different time points (2 h, 3 h, 4 h, 5 h and 6 h) at 37 °C temperature and MTT assay was carried out.

2.7. Determination of optimum dose and time dependent activity of PNP

To evaluate the PNP mediated optimum environments for the defense of cells, hepatocytes were incubated at 37 °C with Ind (200 μM) and various doses of PNP (5, 10, 15, 20, 25 and 30 μg/ml) simultaneously. After that MTT assay has been performed (Mosmann, 1983).

To determine the time-dependent effect of PNP, hepatocytes (containing 1 ml cell suspension ~1 × 10^6 in each) were incubated with Ind at a dose of 200 μM and followed by PNP (at optimum dose) for different time periods (2 h, 3 h, 4 h, and 5 h) at 37 °C temperature. After that MTT assay has been carried out as described earlier.