International Immunopharmacology 39 (2016) 34–40

Contents lists available at ScienceDirect

International Immunopharmacology

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

Impact of MAPK and PI3K/AKT signaling pathways on Malabaricone-A induced cytotoxicity in U937, a histiocytic lymphoma cell line

Alak Manna [a,1], Sritama De Sarkar [a,1], Soumita De [a], Ajay K. Bauri [b], Subrata Chattopadhyay [b], Mitali Chatterjee [a,*]

[a] Dept. of Pharmacology, Institute of Postgraduate Medical Education and Research, 244B, Acharya JC Bose Road, Kolkata 700 020, India
[b] Bio-Organic Division, Bhabha Atomic Research Centre, Trombay, Mumbai 400 085, India

ARTICLE INFO

Article history:
Received 29 March 2016
Received in revised form 1 July 2016
Accepted 4 July 2016
Available online 9 July 2016

Keywords:
Malabaricone-A
MAPK signaling
PI3K/AKT signaling
Pro-oxidant
Reactive oxygen species

ABSTRACT

Intrinsically cancer cells have higher basal levels of reactive oxygen species (ROS), which when augmented by pro-oxidants such as Malabaricone-A (MAL-A) triggers apoptotic cell death, secondary to 'turning on' of the apoptosis related cell signaling pathways. The effects of MAL-A upon key inflammation related signaling molecules were evaluated by western blotting in U937, a histiocytic lymphoma derived cell line. The impact of inhibitors of the pro-apoptotic MAPK and anti-apoptotic PI3K/AKT signaling pathways upon MAL-A induced cytotoxicity and generation of ROS was evaluated by a cell viability assay and flow cytometry respectively in two hematopoietic cell lines, U937 and MOLT3. MAL-A enhanced phosphorylation of the components of the pro-apoptotic pathway, namely ASK1, p38 and JNK. Alongside, MAL-A decreased the phosphorylation of AKT and mTOR. The cytotoxicity of MAL-A was attenuated by inhibitors of p38 and JNK, whereas its cytotoxic potential was enhanced in the presence of a PI3K/AKT inhibitor. Similarly, MAL-A mediated generation of ROS was decreased by inhibitors of p38MAPK and JNK, whereas the PI3K/AKT inhibitor potentiated its generation of ROS. Taken together, MAL-A mediated its cytotoxicity by enhanced generation of ROS via modulation of the apoptosis related cellular signaling pathways and tilting the balance towards a pro-apoptotic scenario. This was achieved via an up-regulation of MAPK (p38 and JNK) along with down-regulation of the PI3K/AKT/mTOR pathway indicating that manipulation of these pathways by compounds such as MAL-A are promising therapeutic targets, worthy of future pharmacological consideration.

1. Introduction

In biological systems, reactive oxygen species (ROS) generated by enzymatic and non-enzymatic reactions play a pivotal role as secondary messengers in signal transduction pathways [1]. The ROS, when released under controlled conditions and in limited amounts, serve as a signaling molecule to promote cellular proliferation and survival [2], via activation of the anti-apoptotic, survival-related signaling pathways namely the phosphatidylinositide 3-kinases (PI3K) and protein kinase B related pathways [3]. Alongside, there is enhancement of the antioxidant components e.g. heme oxygenase, glutathione peroxidase, superoxide dismutase, catalase, glutathione S transferase, NAD(P)H dehydrogenase and quinone 1 among others which collectively support cell proliferation [4]. However, further enhancement of the generation of ROS beyond a critical threshold, triggers activation of the pro-apoptotic signaling pathways, namely forkhead box protein O1 (FOXO1)/TP53, or p53 and mitogen activated protein kinases (MAPKs), culminating in inflammation and cell death [2]. Additionally, associated activation of the pro-apoptotic tumor suppressors, PTEN (phosphatases and tensin homolog) and protein tyrosine phosphatases (PTPs) cause downregulation of the antioxidant system, and the resultant redox imbalance gets translated into an accelerated apoptosis [5].

Malabaricone-A (MAL-A), a diarylnonanoid purified from the fruit rind of *Myristica malabarica* is a potent pro-oxidant that exerted its cytotoxicity towards a panel of leukemic and solid tumor cell lines via induction of a redox imbalance [6,7]. The cytotoxicity was achieved by induction of apoptosis as evident by enhanced annexin V positivity, DNA degradation following inactivation of poly (ADP-ribose) polymerase and arrest of cell cycle progression [7]. Furthermore, MAL-A demonstrated a higher degree of cytotoxicity in three leukemic cell lines *vis-a-vis* solid tumor cell lines, which was mediated following a higher induction of redox imbalance in the former [6]. Accordingly, the focus of this study was to examine the contribution of pro- and anti-apoptosis related signaling pathways in MAL-A mediated cytotoxicity in a histiocytic lymphoma derived cell line.

Abbreviations: ROS, reactive oxygen species; MAPK, mitogen-activated protein kinases; h, hour; BSA, bovine serum albumin; HRP, horseradish peroxidase; TBS, tris-buffered saline; GMFC, geometric mean fluorescence channel.

* Corresponding author.
E-mail addresses: ilatim@vsnl.net, ilatimc@gmail.com (M. Chatterjee).

[1] Should be considered as joint first authors.

http://dx.doi.org/10.1016/j.intimp.2016.07.004
1567-5769/

2. Materials and methods

2.1. Materials

All reagents if not otherwise stated were obtained from Sigma-Aldrich (St. Louis, MO, USA) except Limulus amebocyte lysate (LAL) assay kit (Lonza, Basel, Switzerland), phenazine methosulfate (PMS, Sisco Research Laboratories, Mumbai, India), MTS or 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (Promega, Madison, WI, USA), 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (CMH_2DCFDA, Invitrogen, Carlsbad, CA, USA), antibodies against ASK1, p38MAPK, ERK1/2, JNK, PTEN, AKT, mTOR and their phosphorylated counterpart along with α-tubulin and cell lysis buffer (Cell Signaling Technology Inc., Beverly, MA, USA).

2.2. Malabaricone-A (MAL-A)

Malabaricones (Malabaricone A-D and AL-MAL) were purified from *Myristica malabarica* (Myristicaceae), popularly known as rampatri [8,9]. Mal-A has been characterized by its ^{1}H NMR spectrum [8], while its purity was ascertained by HPLC analysis (RP-18 column, 60:40 acetonitrile/water, flow rate of 1 ml/min., detection at 354 nm) using a JASCO chromatogram (model PU-2080 plus-Intelligent HPLC pump, UV-2075 plus-UV/VIS detector), and showed a single peak at 7.65 min (Fig. 1). They possess a 2-acylresorcinol moiety and their pharmacological activity is based on substitution of their respective aromatic rings [7–9]. The absence of bacterial endotoxin in MAL-A was confirmed by the LAL assay as per manufacturer's instructions. Briefly, samples were incubated serially with LAL and chromogenic substrate. The detection of endotoxin was measured by generation of *p*-nitroaniline at 405 nm and quantified against a standard curve of supplied bacterial endotoxin.

2.3. Cell culture

Two cell lines, U937, a leukemic monocytic lymphoma and MOLT3, an acute lymphoblastic leukemia were maintained in RPMI 1640 medium supplemented with 10% heat inactivated fetal bovine serum, penicillin (50 units/ml), streptomycin (50 μg/ml) and amphotericin-B (1 μg/ml). Cells were sub-cultured every 48–72 h, inoculum being 5×10^5/ml and cell viability (>95%) was confirmed by trypan blue exclusion.

2.4. Immunoblotting

Briefly, U937 cells were resuspended in cell lysis buffer, freeze thawed, centrifuged (12,000 rpm × 10 min, 4 °C) and protein concentration was measured. The samples were resolved in 10% SDS-PAGE and transferred onto nitrocellulose membranes. After blocking the non-specific binding sites with 2% bovine serum albumin (BSA) for 30 min, membranes were incubated overnight at 4 °C with antibodies against ASK1, p38, ERK1/2, JNK, PTEN, AKT, mTOR or their phosphorylated counterpart or alpha-tubulin in TBS containing 2% BSA. Membranes were washed thrice with TBS containing 0.1% Tween 20 and binding was detected with HRP conjugated antibody using a chemiluminescent substrate. Analysis was done using DNR chemiluminescent imaging apparatus [DNR Bio-Imaging Systems Ltd. Jerusalem, Israel] [10]. Each blot was initially probed with the phosphorylated antibody, followed by incubation in a stripping buffer (0.1 M glycine, pH 2.5, 37 °C, 30 min), and then reprobed with its counterpart (total). Finally after another round of stripping, the membranes were reprobed with alpha tubulin as a loading control. The extent of phosphorylation was quantified by densitometric analysis by normalization with respect to their respective total protein.

2.5. Evaluation of Malabaricone-A induced cytotoxicity

The cytotoxicity of MAL-A was evaluated using a formazan based semi-automated MTS/PMS assay as previously described [7]. Briefly, U937 or MOLT3 cells (5×10^4 cells/200 μl of RPMI 1640 medium/well) were pre-incubated for 1 h with inhibitors to p38 (SB 203580, 20 μM), ERK1/2 (PD 98059, 20 μM), JNK (SP 600125, 10 μM) or PI3K/AKT (wortmannin, 0.2 μM) followed by MAL-A (0–100 μg/ml, 48 h at 37 °C/5% CO_2). After 48 h, cell viability was measured as previously described [7]. The IC_{50} i.e. the concentration that inhibited 50% cell growth was enumerated by graphical extrapolation using Graph Pad Prism software (version 5, GraphPad Software Inc., La Jolla, CA, USA).

2.6. Generation of reactive oxygen species (ROS)

The generation of ROS was estimated using a redox-sensitive dye $CM\text{-}H_2DCFDA$ as previously described [7]. Briefly, U937 or MOLT3 cells (5×10^5/well) were pre-incubated for 1 h in the presence of non-toxic concentrations of inhibitors to p38MAPK, ERK1/2, JNK and PI3K/AKT, followed by addition of MAL-A (15 μg/ml, 1 h). Following 2 washes with PBS, the cells were resuspended in PBS containing $CM\text{-}H_2DCFDA$ (2.5 μM) and the fluorescence of CM-DCF was acquired in a flow cytometer.

2.7. Flow cytometry

Cells (5×10^5) from different experimental groups were monitored for their fluorescence on a flow cytometer (FACS Calibur, Becton Dickinson, San Jose, CA, USA) equipped with an argon-ion laser (15 mW)

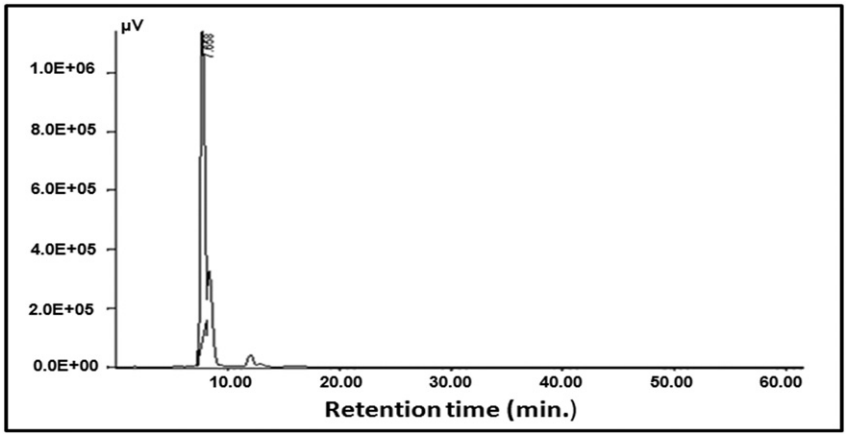

Fig. 1. HPLC profile of MAL-A. Purity of MAL-A were analyzed by HPLC, detecting a single peak at 354 nm at 7.65 min.