Phytomedicine 22 (2015) 713–723

Contents lists available at ScienceDirect

Phytomedicine

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

The variable chemotherapeutic response of Malabaricone-A in leukemic and solid tumor cell lines depends on the degree of redox imbalance

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ARTICLE INFO

Article history:
Received 29 September 2014
Revised 12 May 2015
Accepted 20 May 2015

Keywords:
Apoptosis
Malabaricone-A
Pro-oxidant
Oxidative stress
Reactive oxygen species
Redox imbalance

ABSTRACT

Purpose: The 'two-faced' character of reactive oxygen species (ROS) plays an important role in cancer biology by acting as secondary messengers in intracellular signaling cascades, enhancing cell proliferation and survival, thereby sustaining the oncogenic phenotype. Conversely, enhanced generation of ROS can trigger an oxidative assault leading to a redox imbalance translating into an apoptotic cell death. Intrinsically, cancer cells have higher basal levels of ROS which if supplemented by additional oxidative insult by pro-oxidants can be cytotoxic, an example being Malabaricone-A (MAL-A). MAL-A is a plant derived diarylnonanoid, purified from fruit rind of the plant *Myristica malabarica* whose anti-cancer activity has been demonstrated in leukemic cell lines, the modality of cell death being apoptosis. This study aimed to compare the degree of effectiveness of MAL-A in leukemic vs. solid tumor cell lines.

Methods: The cytotoxicity of MAL-A was evaluated by the MTS-PMS cell viability assay in leukemic cell lines (MOLT3, K562 and HL-60) and compared with solid tumor cell lines (MCF7, A549 and HepG2); further studies then proceeded with MOLT3 vs. MCF7 and A549. The contribution of redox imbalance in MAL-A induced cytotoxicity was confirmed by pre-incubating cells with an antioxidant, *N*-acetyl-L-cysteine (NAC) or a thiol depletor, buthionine sulfoximine (BSO). MAL-A induced redox imbalance was quantitated by flow cytometry, by measuring the generation of ROS and levels of non protein thiols using dichlorofluorescein diacetate (CM-H_2DCFDA) and 5-chloromethylfluorescein diacetate (CMFDA) respectively. The activities of glutathione peroxidase (GPx), superoxide dismutase, catalase (CAT), NAD(P)H dehydrogenase (quinone 1) NQO1 and glutathione-*S*-transferase GST were measured spectrophotometrically. The mitochondrial involvement of MAL-A induced cell death was measured by evaluation of cardiolipin peroxidation using 10-*N*-nonyl acridine orange (NAO), transition pore activity with calcein-AM, while the mitochondrial transmembrane electrochemical gradient ($\Delta\psi_m$) was measured by JC-1, fluorescence being acquired in a flow cytometer. The apoptotic mode of cell death was evaluated by double staining with annexin V-FITC and propidium iodide (PI), cell cycle analysis by flow cytometry and caspase-3 activity spectrophotometrically. The expression of Nrf2 and HO-1 was examined by western blotting.

Results: MAL-A demonstrated a higher degree of cytotoxicity in three leukemic cell lines whose IC_{50} ranged from 12.70 ± 0.10 to 18.10 ± 0.95 μg/ml, whereas in three solid tumor cell lines, the IC_{50} ranged from 28.10 ± 0.58 to 55.26 ± 5.90 μg/ml. This higher degree of cytotoxicity in MOLT3, a leukemic cell line was due to a higher induction of redox imbalance, evident by both an increased generation of ROS and concomitant depletion of thiols. This was confirmed by pre-incubation with NAC and BSO, wherein NAC decreased MAL-A induced cytotoxicity by 2.04 fold while BSO enhanced MAL-A cytotoxicity and decreased the IC_{50} by 5.60 fold. However, in solid tumor cell lines (MCF7 and A549), NAC minimally decreased MAL-A induced cytotoxicity, and BSO increased the IC_{50} by 1.96 and 2.39 fold respectively. Furthermore, the generation of ROS by MAL-A increased maximally in MOLT3 as the fluorescence increased from 44.28 ± 7.85 to 273.99 ± 32.78, and to a lesser degree in solid tumor cell lines, MCF7 (44.28 ± 14.89 to 207.97 ± 70.64) and A549 (37.87 ± 3.24 to 147.12 ± 38.53). In all three cell lines there was a concomitant depletion of thiols as in MOLT3, the GMFC decreased from 340.65 ± 60.39 to 62.67 ± 11.32, in MCF7 (277.82 ± 50.32 to 100.39 ± 31.93) and in A549 (274.05 ± 59.13 to 83.15 ± 21.43). In MOLT3 as compared to MCF7 and A549, decrease in the

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http://dx.doi.org/10.1016/j.phymed.2015.05.007

activities of GPx, CAT, NQO1 and GST was substantially greater. In all cell lines, the MAL-A induced redox imbalance translated into triggering of initial mitochondrial apoptotic events. Here again, MAL-A induced a higher degree of cardiolipin peroxidation in MOLT3 (67.01%) than MCF7 and A549 (29.15% and 44.30%), as also down regulated the mitochondrial transition pore activity from baseline to a higher extent, GMFC being 48.05 ± 2.37 to 10.70 ± 3.97 (MOLT3), 43.55 ± 3.36 to 15.36 ± 0.60 (MCF7) and 39.58 ± 0.4 to 12.65 ± 1.56 (A549). Perturbation of mitochondrial membrane potential evident by a decrease in the ratio of red/green (J-aggregates/monomers) was 134 fold (14.73/0.11) in MOLT3, 45 fold in MCF7 (20.72/0.46) and 34 fold in A549 (22.01/0.64). The extent of apoptosis using a similar concentration of MAL-A was maximal in MOLT3, wherein a 105 fold increase in annexin V binding was evident (0.83 ± 0.51 to 87.08 ± 9.85%) whereas it increased by 43.11 fold in MCF7 (0.69 ± 0.30 to 29.75 ± 11.79%) and 47.52 fold in A549 (0.61 ± 0.31 to 28.99 ± 17.21%). MAL-A induced apoptosis was also associated with a higher degree of caspase-3 activity in MOLT3 vs. MCF7 or A549 which translated into halting of cell cycle progression, evident by an increment in the sub-G_0/G_1 population [19.26 fold in MOLT3 (0.95 ± 0.45 vs. 18.30 ± 1.90%), 11.01 fold in MCF7 (0.97 ± 0.37 vs. 10.68 ± 0.69%) and 8.58 fold in A549 (1.06 ± 0.45 vs. 9.10 ± 1.05%)]. MAL-A effectively inhibited Nrf2 and HO-1, more prominently in MOLT3. Furthermore, the decreased expression of Nrf2 in MOLT3 correlated with the decreased activities of NQO1 and GST, suggesting that targeting of the Nrf2 anti-oxidant pathway could be considered.
Conclusion: Taken together, MAL-A a pro-oxidant compound is likely to be more effective in leukemias, meriting further pharmacological consideration.

Introduction

Reactive oxygen species (ROS) are considered as a double-edged sword owing to their ability to initiate cancer progression and conversely mediate prevention. At low doses, ROS especially H_2O_2 are mitogenic and promotes cell proliferation, intermediate doses cause growth arrest, whereas higher doses inflict a severe degree of oxidative stress culminating in cell death (Yang et al. 2013). Therefore, tumor cells can be targeted by decreasing levels of ROS or mounting an additional oxidative assault beyond the critical threshold (Mao et al. 2014) and indeed, this is emerging as a promising anti-cancer strategy (Yang et al. 2013).

This ability to increase intrinsic ROS has been exploited in the management of hematological malignancies and demonstrated by several plants derived natural products (Dassprakash et al. 2012). Arsenic trioxide has been extensively evaluated in leukemias and solid tumors (Dilda and Hogg 2007), wherein its IC_{50} in leukemias ranged from 1 to 2 μM (Bornhauser et al. 2007); however, in solid tumor cell lines, the IC_{50} ranged from 2 to 50 μM (Kotowski et al. 2012).

Malabaricone-A (MAL-A), a plant derived diarylnonanoid, purified from fruit rind of the plant *Myristica malabarica*, demonstrated pro-oxidant activity in leukemic cell lines, with cell death being via increased apoptosis (Manna et al. 2012). However, its effectiveness in solid tumor malignancies has not been evaluated and accordingly, in this study, we compared the effect of MAL-A on the redox status and induction of apoptosis in leukemic vs. solid tumor cell lines.

Materials and methods

Malabaricone-A (MAL-A)

Malabaricones (Malabaricone A-D and AL-MAL) sourced from the Western Ghats of Karnataka, India were purified from *Myristica malabarica* (Myristicaceae), popularly known as rampatri, Bombay mace or false nutmeg (Patro et al. 2005). They possess a 2-acylresorcinol moiety and differ in the substitution of their respective aromatic rings, that impacts substantially on its pro-oxidant activity (Manna et al. 2012).

Cell culture

Human leukemic and solid tumor cell lines (Table 1) were maintained at 37 °C, 5% CO_2 in RPMI1640 medium, except for HepG2 that was maintained in Dulbecco's modified eagle's medium. Both media were supplemented with 10% heat inactivated fetal bovine serum, penicillin (50 units/ml), streptomycin (50 μg/ml) and amphotericin-B (1 μg/ml).

In vitro evaluation of cytotoxic activity of MAL-A

The effect of MAL-A was evaluated using a formazan based semi-automated MTS/PMS assay (Manna et al. 2012).

Effect of MAL-A on the redox status

The generation of ROS and non protein thiols after treatment with MAL-A (15 μg/ml, 0–12 h) was estimated by flow cytometry using dichlorofluorescein diacetate (CM-H_2DCFDA, 2.5 μM) and 5-chloromethylfluorescein diacetate (CMFDA, 0.5 μM) respectively. The activity of glutathione peroxidase (GPx) (Manna et al. 2012), superoxide dismutase (SOD, Marklund and Marklund 1974) and catalase (Beers and Sizer 1952) was measured spectrophotometrically.

Determination of NQO1 and GST was performed as previously described (Wondrak 2007; Borges et al. 2013). Briefly, cells treated with MAL-A (15 μg/ml, 0–12 h) were lysed in ice cold buffer (0.1 M phosphate buffer, 0.1% Tween 20, pH 7.0). After cell disruption (Manna et al. 2012), the cell lysates (50 μg) were incubated with the reaction mixture (1 ml) containing 25 mM Tris–HCl (pH 7.4), 180 μM NADPH, BSA (0.2 mg/ml), Tween 20 [0.01% (v/v)]. For NQO1 activity, the reaction was started by addition of 2,6-dichlorophenolindophenol (DCPIP, 20 mM stock in DMSO, 2 μl) in the absence or presence of dicoumerol (20 μM). Absorbances were measured for 1 min at 600 nm ($\varepsilon_{DCPIP} = 21 \times 10^3$ M^{-1} cm^{-1}) and the NQO1 activity expressed as μmol DCPIP/mg protein/min. For determination of GST activity, cell lysates (50 μg) were incubated with glutathione (GSH, 1.5 mM), CDNB (0.2 mM in 0.1 M phosphate buffer, pH 7.0) and absorbances at 340 nm were immediately measured for 3 min every 30 s. GST activity was determined using the molar extinction coefficient ($\varepsilon = 9.6 \times 10^3$ mM^{-1} cm^{-1}).

Analysis of mitochondrial apoptotic events

The effect of MAL-A (15 μg/ml, 1 h, 37 °C) on the mitochondrial transmembrane electrochemical gradient ($\Delta\psi_m$) and cardiolipin peroxidation was measured using JC-1 (7.5 μM, 10 min, 37 °C) and 10-*N*-nonyl acridine orange (NAO, 100 nM, 37 °C, 10 min, Manna et al. 2012) respectively. Similarly, the transition pore activity was measured using Calcein-AM (10 nM, 37 °C, 15 min, Martinez-Abundis et al. 2012) and fluorescence was acquired in a flow cytometer.