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Effectiveness of malabaricone-A in P-glycoprotein over-expressing cancer cell lines

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ABSTRACT

Background: A major impediment in treatment for cancers is resistance to chemotherapy and is primarily attributed to over-expression of efflux pumps. This study aimed to establish the cytotoxicity of malabaricone-A (MAL-A) in P-glycoprotein/multidrug resistance (P-gp/MDR) over-expressing hematopoietic cancer cell lines.

Methods: Leukemia and multiple myeloma cell lines were indirectly evaluated for their P-gp/MDR status by examining Calcein-AM fluorescence and cell viability was assessed by the MTS-PMS assay.

Results: The fluorescence of calcein was significantly decreased in three cell lines LP-1, RPMI-8226 and CEM-ADR 5000 and reversal with verapamil endorsed their P-gp/MDR activity. The mean IC50 of MAL-A in these MDR+ cell lines (5.40 ± 1.41 to 12.33 ± 0.78 µg/ml) was comparable with the MDR-leukemic (9.72 ± 1.08 to 19.26 ± 0.75 µg/ml) and multiple myeloma cell lines (9.65 ± 0.39 to 18.05 ± 0.17 µg/ml).

Conclusions: Irrespective of their P-gp activity, the cytotoxicity of MAL-A was comparable, making it worthy of future pharmacological consideration in multidrug resistance.

Keywords: Anti-cancer, Calcein, Malabaricone-A, Multidrug resistance MDR, P-glycoprotein

INTRODUCTION

Resistance of tumors to chemotherapeutic agents is an important factor that limits the successful treatment of a wide range of malignancies including hematological malignancies. This is often attributed to an over expression of permeability glycoprotein (P-gp), an efflux pump belonging to the family of ATP-binding cassette (ABC) family of drug transporters.[1]

The expression of P-gp has been correlated with drug sensitivity and clinical outcome in several studies pertaining to acute myelogenous leukemias (AML), multiple myelomas (MM), and malignant lymphomas.[2] Numerous structurally and functionally unrelated anticancer drugs, e.g. doxorubicin, vincristine and methotrexate have been shown to be substrates of P-gp but despite promising preclinical data, the inhibitors of P-gp have failed to translate into clinical benefit.[1] Therefore, the

search for cytotoxic compounds that are effective in MDR cancers is ongoing.

The plant *Myristica malabarica* (Myristicaceae), commonly known as Rampatri, Bombay mace, or false nutmeg) is an exotic spice in various Indian cuisines. From the fruit rind of Rampatri, phytoconstituents have been sourced that have anti-cancer effects.[3] Among the phytoconstituents, MAL-A, MAL-B and MAL-D have demonstrated cytotoxicity in leukemia cell lines.[4-6]

As MAL-A has been shown to be effective in a P-gp over-expressing MDR+ cancer cell line, this study was extended to a broader panel of leukemic and multiple myeloma cell lines.[6] Accordingly, the P-gp status was identified within representative leukemia and multiple myeloma cancer cell lines and the cytotoxicity of MAL-A was evaluated in these MDR+ and MDR- cell lines.

METHODS

Reagents

All chemicals if not stated otherwise were obtained from Sigma-Aldrich (St. Louis, MO, USA) except phenazine-methosulphate (PMS), from Sisco Research Laboratories (Mumbai, Maharashtra, India), MTS or 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (Promega, Madison, WI, USA) and calcein AM from Invitrogen (Carlsbad, CA, USA).

Isolation of malabaricone-A (MAL-A)

Malabaricone-A, a diarylnonanoid was isolated from a methanolic extract of the fruit rind of *Myristica malabarica* as previously described.[7] Briefly, the dry fruit rinds of *M. malabarica* were powdered in a grinder and extracted with methanol (60 mL×4 days at room temperature).

The supernatants were decanted and after repeating the entire process thrice, the combined supernatants were filtered, and evaporated at <40ºC in vacuo to obtain the methanolic extract which was stored in a vacuum desiccator.

To isolate the chemical constituents, a part was subjected to partial fractionation using a silica gel (25 g) column and eluted with hexane to collect five 300 ml fractions. These on evaporation in vacuo were fully characterized using IR and 1H NMR spectroscopic data.[7]

Cell culture

Human leukemic and multiple myeloma cell lines along with a pair of doxorubicin sensitive and resistant leukemia cell lines were used in this study (Table 1). The cell lines were maintained in RPMI 1640 medium (pH 7.4) supplemented with 10% heat inactivated fetal bovine serum (FBS), penicillin (50 units/ml), streptomycin (50 µg/ml) and amphotericin-B (1 µg/ml) at 37ºC in a humidified incubator containing 5% CO_2.

The CEM ADR 5000 cell line was generated from the parent drug sensitive CCRF-CEM cell line by addition of doxorubicin (5.0 µg/ml, 24 hours) every 7 days, and was demonstrated to over express P-glycoprotein.[8] Cells were sub-cultured every 48-72 hours, with an inoculum of 5 x 10^5/ml and cell viability (>95%) was confirmed by trypan blue exclusion.

Determination of optimal concentrations of calcein-AM and verapamil

The concentration of calcein-AM that would be effective for measurement of P-gp activity was determined by incubating CCRF-CEM cells (5 x 10^5 cells/tube) for 30 minutes at 37ºC with increasing concentrations of calcein-AM (0-100 nM) and fluorescence measured by flow cytometry.

Table 1: Human leukemic and multiple myeloma cell lines used in this study.

Name	Cell type	Tissue	Disease
U937	Monocytes	Pleural effusion	Histiocytic lymphoma
MOLT3	T-lymphoblast	Peripheral blood	Acute lymphoblastic leukemia (ALL)
CCRFCEM	T-lymphoblast	Peripheral blood	ALL (doxorubicin sensitive)
CEM ADR 5000	T-lymphoblast	Peripheral blood	ALL (doxorubicin resistant)
K562	Lymphoblast	Bone marrow	Chronic myeloblastic leukemia
RAJI	B-lymphoblast	Burkitt lymphoma	Burkitt lymphoma
LP-1	Plasma cell	Peripheral blood	Multiple myeloma
MOLP-8	Plasma cell	Peripheral blood	Multiple myeloma
OPM-2	Plasma cell	Peripheral blood	Multiple myeloma
KMS-12BM	Plasma cell	Peripheral blood	Multiple myeloma
RPMI-8226	Plasma cell	Peripheral blood	Multiple myeloma

As there was no increase in fluorescence beyond 10 nM, this concentration was selected for all subsequent experiments. For standardization of verapamil, the P-gp over expression was studied in the CEM ADR 5000 cell line. Briefly, cells (1 x 10^6 cells/ml in medium) were incubated with varying concentrations of verapamil (3 hours, 37ºC). Following two washes with PBS, calcein-AM (10 nM) was added for 30 minutes at 37ºC and fluorescence acquired. Fluorescence plateaued with 100 µM verapamil and accordingly, was selected for all subsequent experiments.

Measurement of P-gp activity

Cell lines were monitored for their intracellular calcein fluorescence, briefly cells (1 x 10^6/ml RPMI medium containing 5% FBS) were incubated in the absence and presence of verapamil (100 µM) for 3 hours at 37ºC. After two washes in PBS, cells were incubated with calcein-AM (10 nM in PBS) for 30 minutes at 37ºC and the resultant fluorescence was acquired.

Evaluation of cytotoxicity of malabaricone-A in human hematopoietic cell lines

The cytotoxicity of MAL-A was evaluated using a formazan based semi-automated MTS/PMS assay.[4] Briefly, cells (5 x 10^4/200 µl of medium/well) were incubated with MAL-A (0-100 µg/ml) for 48 hours at 37ºC and 5% CO_2. After 48 hours incubation, MTS (2 mg/ml in PBS) and PMS (0.92 mg/ml in PBS) in 10:1 ratio (20 µl per well) was added, plates were incubated in the dark for 3 hours at 37ºC and optical densities were measured at 490 nm (O.D 490, Spectra Max M2e, Molecular Devices, Sunnyvale, CA, USA). The specific absorbance representing formazan production was calculated by subtraction of background absorbance from total absorbance and the mean %viability was calculated.

Mean specific O.D490 of MAL-A treated cells × 100/Mean specific O.D. 490 of untreated cells.

Each experiment was repeated thrice in duplicates, the data obtained was plotted and the IC50 i.e. the concentration inhibiting 50% cell growth calculated by graphical extrapolation using the Graph Pad Prism software (version 5, La Jolla, CA, USA).

Flow cytometry

The fluorescence of 10,000 cells was acquired by flow cytometry initially using a bivariate FSC vs SSC plot to gate the population and a FL1 histogram to quantify the fluorescence. Finally, analysis was made by BD Cell Quest Pro software (BD Biosciences, San Jose, CA, USA) to detect geometrical mean fluorescence channel (GMFC).

Each experiment was performed at least thrice in duplicates and results expressed as mean±SEM. Statistical analysis was evaluated by Students t-test/Mann Whitney

test (wherever applicable), using Graph Pad Prism software, version 5 (La Jolla, CA, USA), p <0.01 was considered as statistically significant.

RESULTS

Isolation and characterization of malabaricone-A

Different extracts were initially prepared by extracting Rampatri successively with ether, methanol and water.[7] The methanolic extract was further fractionated into five fractions (F1-F5) of different polarities. From the most active fraction (F2), five compounds were isolated and characterized by spectroscopy and amongst these compounds 2-5 were reported as malabaricones A-D.[7] (Figure 1). In view of malabaricone-A (MAL-A) demonstrating cytotoxicity in leukemic cell lines as also pronounced generation of reactive oxygen species, further studies were undertaken with MAL-A and this study was also undertaken using MAL-A.[4-6]
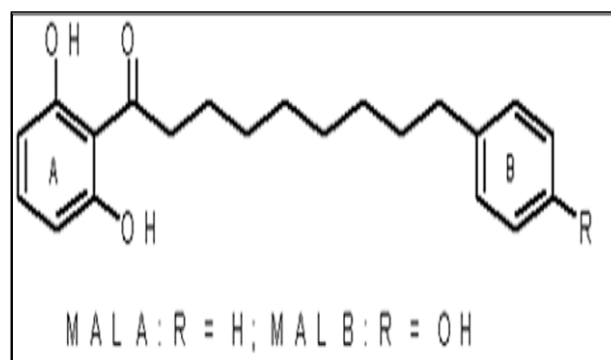

**Figure 1: Chemical structure of malabaricones isolated from the methanolic extract of Rampatri.**

Determination of P-gp activity in human hematopoietic cancer cell lines

Calcein-AM is a high affinity, non-fluorescent substrate of the MDR1 transporter which rapidly penetrates the cell membrane and gets trapped intracellularly upon conversion into fluorescent calcein by non-specific cytoplasmic esterases. In MDR1 expressing cells, calcein-AM is rapidly extruded by P-gp, before its intracellular conversion to calcein, resulting in a decreased fluorescence.[9,10]

However, when the MDR1 pump activity is blocked by P-gp inhibitors e.g. verapamil, the fluorescent free calcein now rapidly accumulates, leading to an increase in fluorescence. Accordingly, the fluorescence of calcein is considered as a quantitative measurement of MDR1 activity.[11]

The P-gp pump status in terms of calcein fluorescence was evaluated in leukemic and multiple myeloma cell lines in the absence and presence of a P-gp blocker verapamil. The CEM ADR5000 was taken as the representative MDR cell line whose calcein fluorescence was 31.5-fold lower than

its sensitive counterpart CCRF CEM, being 13.11±1.46 vs 412.9±13.11 (Figure 2 and Table 2).
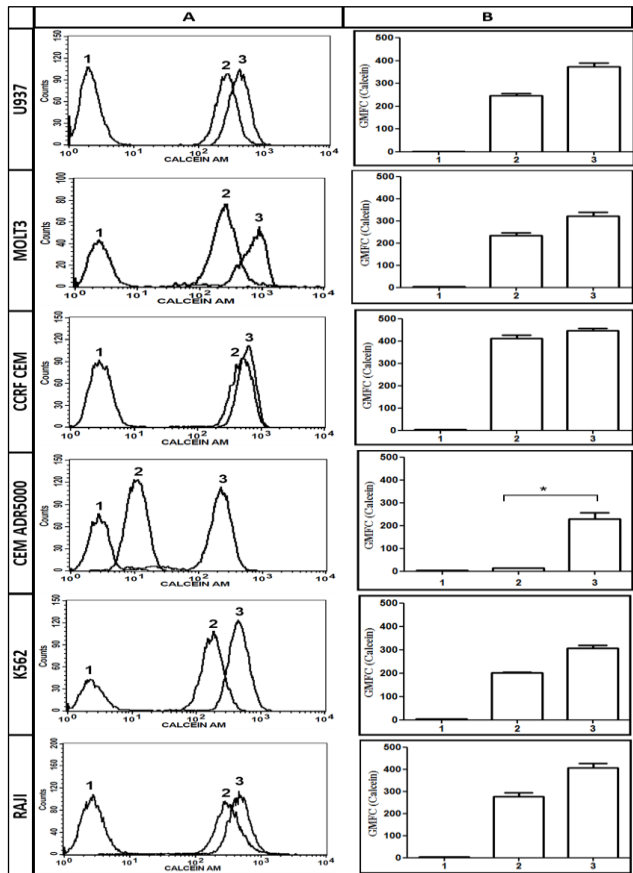
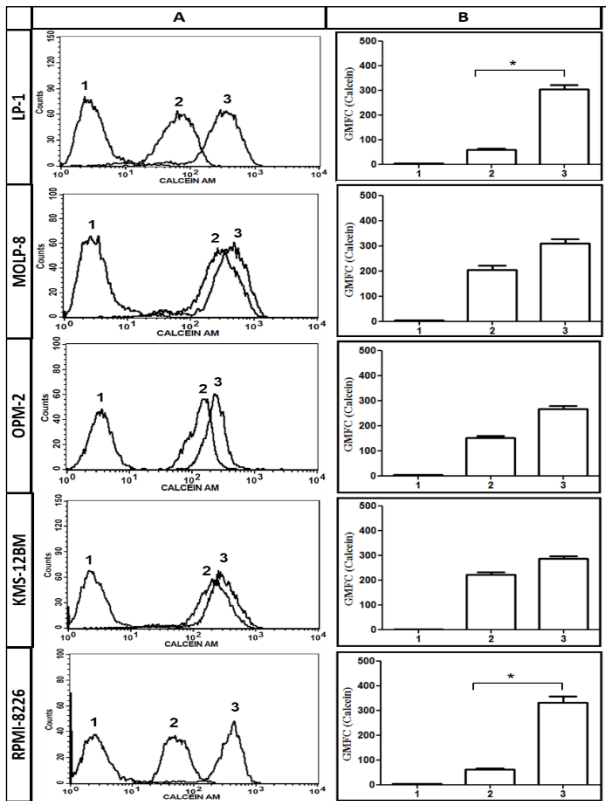*p <0.01 significantly different from its baseline.

Figure 2: Status of calcein fluorescence in leukemic cell lines. A) Representative flow cytometric histogram profiles of calcein fluorescence in leukemic cell lines. B) Effect of verapamil on calcein fluorescence.

Cells (1 x 10^6/ml, 1, unstained) were incubated in the absence (2) and presence of verapamil 100 µM (3) for 3 h followed by labelling with Calcein-AM (10 nM) (Figure 2A). Cells (1 x 10^6/ml, 1, unstained) were incubated in the absence (2) and presence of Verapamil 100 µM (3) for 3 hours after which the fluorescence of calcein (10 nM) was measured and represented as mean GMFC±SEM (Figure 2B). With the addition of verapamil, there was a 17.5-fold increase in GMFC, corroborative of its enhanced P-gp/MDR status, as also confirmed the assay specificity (Table 2).[8] The basal fluorescence generated in the leukemic cell lines U937, MOLT3, K562 and RAJI ranged from 201.7 to 247.2 and was comparable with the drug sensitive CCRF CEM cell line; with the addition of verapamil, there was a marginal alteration in fluorescence in these 4 cell lines and therefore confirmed their low expression of P-gp (Figure 2 and Table 2). Cells (1 x 10^6/ml), were incubated in the absence and presence of Verapamil (100 µM) for 3 h followed by labelling with Calcein-AM (10 nM) and GMFC was measured as described in Materials and methods. Data are expressed as mean GMFC ± SEM of at least three experiments in duplicate (Table 2).

In the myeloma cell lines, the basal fluorescence ranged from 60.08 to 221.4, with the addition of verapamil, there was a minimal change in fluorescence in MOLP-8, OPM-2 and KMS-12BM cell lines (Figure 3 and Table 2). However, in LP-1 and RPMI-8226, the addition of verapamil resulted in a 5.0- and 5.5-fold increase in fluorescence respectively, indicating these two cell lines have increased P-gp activity (Figure 3 and Table 2). The concentration of verapamil used was non-toxic in all cell lines as confirmed by propidium iodide exclusion, positivity being <5%. The auto-fluorescence was minimal, indicating that the observed increase in fluorescence was attributable to its P-gp pump activity, which was inhibited by verapamil.

Table 2: Evaluation of P-gp activity in hematopoietic cell lines.

Name	GMFC (Calcein)		Fold increase in fluorescence
Leukemic cell lines	-Verapamil	+Verapamil	
U937	247.2±8.99	373.4±16.20	1.51
MOLT3	234.9±12.13	321.6±17.54	1.36
CCRF-CEM	412.9±13.11	446.6±8.91	1.08
CEM ADR5000	13.11±1.46	229.0±27.09	17.46
RAJI	277.4±16.91	405.5±21.59	1.46
K562	201.7±2.96	305.8±14.48	1.52
Multiple Myeloma cell lines			
LP-1	60.08±4.52	305.2±16.65	5.08
MOLP-8	205.3±16.55	310.0±16.03	1.50
OPM-2	150.6±7.37	265.6±12.73	1.76
KMS-12BM	221.4±10.65	287.2±10.47	1.29
RPMI-8226	60.57±6.92	330.9±25.09	5.46

*p< 0.01 significantly different from its baseline.

Figure 3: Status of calcein fluorescence in multiple myeloma cell lines. A) Representative flow cytometric histogram profiles of Calcein fluorescence in multiple myeloma cell lines. B) Effect of Verapamil on Calcein fluorescence.

Cells (1 x 106/ml, 1, unstained) were incubated in the absence (2) and presence of Verapamil 100 µM (3) for 3 h followed by labelling with Calcein-AM (10 nM) (Figure 3A). Cells (1 x 106/ml, 1, unstained) were incubated in the absence (2) and presence of Verapamil 100 µM (3) for 3 h, after which the fluorescence of Calcein (10 nM) was measured and represented as mean GMFC±SEM (Figure 3B). Accordingly, the cell lines were grouped based on their P-gp activity, in that a 3-fold or higher increase in calcein fluorescence following the addition of verapamil was considered as a MDR+ cell line (Tables 2 and 3) and comprised of CEM ADR5000, LP-1 and RPMI-8226. In the remaining eight cell lines, where there was no increase in calcein fluorescence, and were considered as MDR- (Tables 2 and 3).

Determination of IC50 of Malabaricone-A in human hematopoietic cancer cell lines

The crude extract Rampatri and the derived phytoconstituents, namely malabaricones, MAL-A, MAL-B and MAL-D have been reported to be cytotoxic in leukemic cell lines.[4,5] As MAL-A and MAL-D were comparable as regards their cytotoxicity, MAL-A was studied as a representative compound. The cytotoxicity of Malabaricone-A (MAL-A) was tested in a broad panel of human leukemia and multiple myeloma cell lines (Table 1). The mean IC50 of MAL-A in MDR- cell lines ranged from 9.72±1.08 to 19.26±0.75 µg/ml, while in the MDR+ cell lines, the IC50 of MAL-A was comparable and ranged from 5.40 ± 1.41 to 12.33±0.78 µg/ml (Table 3 and Figure 4).
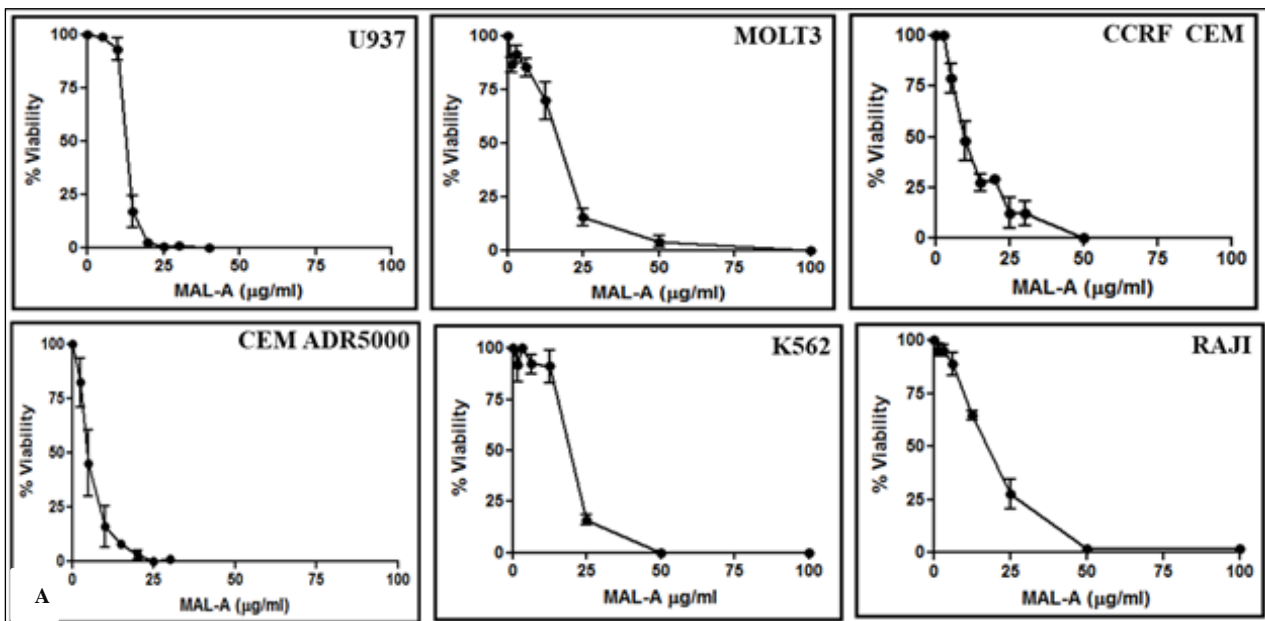

Figure 4: Cytotoxicity (IC50) of MAL-A in MDR+ and MDR- hematopoietic cell lines. Cells (5x10⁴/200 µl) from six leukemic (A) five multiple myeloma cell lines.
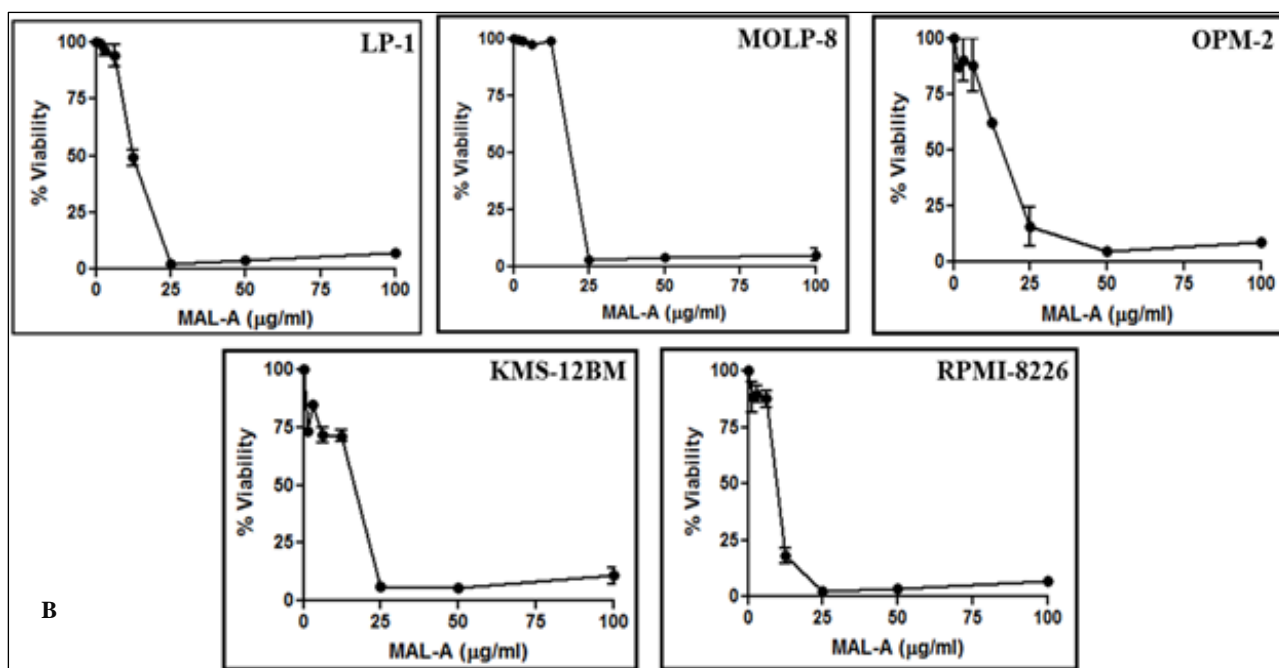
Figure 4: Cytotoxicity (IC50) of MAL-A in MDR+ and MDR- hematopoietic cell lines. Cells (5x10⁴/200 µl) from six leukemic (B) were incubated with MAL-A (0-100 µg/ml) for 48 hours and cell viability was measured by MTS-PMS assay as described in Materials and methods. Each data is the mean IC50 (mean±SEM) derived from at least three experiments in duplicate.

DMSO (0.2%), representative of the highest concentration present in MAL-A (100 µg/ml) did not alter cell viability, confirming its biological inertness. Cells (5 x 10⁴/200 µl) from leukemic* and multiple myeloma# cell lines were treated with MAL-A (0-100 µg/ml) for 48 h and cell viability was measured by the MTS-PMS assay as described in Materials and methods. Each data is the mean IC50 (mean±SEM) derived from at least three experiments in duplicate (Table 3).

Table 3: Cytotoxicity of MAL-A in MDR+ and MDR- hematopoietic cell lines.

Cell line	IC$_{50}$ (µg/ml)
MAL-A in MDR-	
*CCRF-CEM	9.72±1.08
*MOLT3	17.20±2.22
*U937	12.75±0.46
*K562	19.26±0.75
*RAJI	17.00±1.37
#MOLP-8	18.05±0.17
#OPM-2	15.85±1.15
#KMS-12BM	16.70±0.36
MAL-A in MDR+	
*CEM ADR5000	5.40±1.41
#LP-1	12.33±0.78
#RPMI-8226	9.65±0.39

DISCUSSION

Cancer is the leading cause of death in economically developed countries and the second leading cause of death in developing countries. Global cancer burden is estimated to have escalated to 18.1 million new cases and 9.6 million deaths in 2018. According to the World Health Organization (WHO), by the year 2050, it is expected that 27 million new cancer cases and 17.5 million cancer deaths will occur per year.[12]

Cancer chemotherapy is aimed to achieve and maintain remission in the treatment of hematological malignancies wherein MDR to chemically un-related anticancer drugs poses a formidable challenge in cancer treatment. The most widely studied MDR mechanisms involve drug efflux mechanisms where ATP-binding cassette (ABC) membrane transporters play a contributory role and include P-glycoprotein (P-gp, ABCB1, MDR1), multidrug resistance protein 1 (MRP1, ABCC1) and breast cancer resistance protein (BCRP, ABCG2).[13-15] Numerous anticancer drugs, including agents commonly used for the treatment of hematological malignancies e.g. doxorubicin, vincristine, and methotrexate have been shown to be P-gp substrates, and clinical studies have demonstrated a strong association between P-gp expression and response to therapy or survival rates in acute leukemias and multiple myelomas.[1,16]

To overcome MDR, strategies include bypassing of recognition, blockade of efflux pumps, modification of essential growth molecules among others. Studies have explored the ability of improving chemotherapeutic efficacy by suppressing or evading MDR mechanisms including the development of new anticancer drugs that can escape the efflux pumps, MDR modulators or chemosensitizers, multifunctional nanocarriers, and RNAi therapy.[17,18] Promising preclinical data of P-gp inhibitors for overcoming MDR have unfortunately not translated into clinical benefit.[19,20]

The effectiveness of MAL-A, a diarylnonanoid isolated from the methanolic extract of Mysristica malabarica (Figure 1) in a representative MDR cancer cell line has been evaluated, and therefore was extended to a larger panel of hematopoietic cancer cell lines (Table 2 and Figures 2, 3).[6] The P-gp activity was found to be minimal in the leukemic cell lines studied whereas in the multiple myeloma cell lines, 2/5 showed strong P-gp activity (Table 2). In clinical studies, the occurrence of MDR in multiple myeloma has been reported (Abraham J et al,) and this study corroborated the same.[16] MAL-A was found to be cytotoxic in all the multiple myeloma cell lines irrespective of their levels of P-gp activity (Figure 4 and Table 3). Taken together, MAL-A, a plant derived compound, demonstrated similar chemosensitivity in multidrug resistant and drug sensitive cell lines, suggesting its potential application in overcoming drug resistance. Further evaluation in animal models of cancer and chemoresistant cancers e.g. solid tumors are warranted to exploit the promising ability of MAL-A in overcoming the adverse prognostic impact of multidrug resistance.

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