Abstract

Cancer is one of the leading causes of deaths all over the world. The search for the novel drugs is essential for chemotherapy, due to inherent or acquired resistance in cancer cells against conventional chemotherapy. Dysregulation in apoptotic pathways, improved DNA repair, drug transport and detoxification are considered to be the major causes of drug resistance. There is a burgeoning interest in small natural organic molecules, capable of switching their redox status, as these can often cleave DNA and/or target mitochondria, and hence can be used as anti-cancer agents. To this end, this review describes the discovery of an important polyphenolic viz. malabaricone C (Mal C), which was isolated from fruit rind of rampatri spice, possessing impressive anti-cancer properties in vitro and in vivo tumor models. Mal C shows higher therapeutic efficacy than curcumin and resveratrol against multiple cancer cells. This review also highlights synthesis and mechanistic evaluation of mal C and its analogue for their anticancer potential.

Keywords: Myristica malabarica, Malabaricones, Cancer therapy, Redox regulation, Mitochondrial dysfunction

Introduction

Cancer is one of the leading causes of mortality in developing countries with lung and breast cancer contributing maximum cancer related mortalities in men and women, respectively [1]. Lungs and breast cancer contributes to 5.9% and 14% of new cases of cancers per year in India, and accounts for 19% of cancer related deaths worldwide [1]. Apart from primary lung cancers, lungs are the second most common sites for metastatic spread [2]. In particular, the aggressive and highly metastatic late stage melanoma is well-known to induce secondary lung cancers. The 5-year survival rate is less than 15% in patients with metastatic disease and an approximately one-third of all melanoma patients experience disease recurrence [3]. Various chemotherapeutic agents are being exploited for management of lung cancer, breast cancer and metastasis. However, systemic hemato-and-neuro-toxicity/side-effect and cancer recurrence because of inherent or acquired resistance remain major issues in cancer therapy [4]. Drugs that can modulate the actions of...
Multiple targets may reduce/overcome the chances of drug resistance and recurrence in cancer therapy.

Current epidemiological evidences show a strong influence of specific diet on cancer prevention. Extensive work is also being pursued to identify compounds from dietary and medicinal plants and other natural resources, as these are popularly believed to be non-toxic to humans [5]. Also, an approximately 50% of the internationally approved chemotherapeutic drugs are derived from natural products [6]. Spices are present in most of our daily diets, and consumption of spice rich food has been found to play an important role in suppressing the transformative, hyper-proliferative and inflammatory processes that initiate carcinogenesis [7]. The fruit rind of the plant Myristica malabarica (Myristicaceae) (popularly known as rampatri, Bombay mace, or false nutmeg) is used as an exotic spice in various Indian cuisines. This is credited with hepatoprotective, anticarcinogenic, and antithrombotic properties and is found as a constituent in many Ayurvedic preparations such as pasupasi. Several herbal formulations containing M. malabarica are also claimed to possess antitumor effect [8]. However, most of the medicinal attributes of the spice have not been substantiated adequately. Earlier, we have found that amongst the four malabaricones A–D (designated as mal A–D), isolated from its extract, mal B and mal C possess superior antioxidant [8], anti-inflammatory [9], anti-ulcer [10, 11] and cardioprotective properties [12]. The chemical structures of mal A–D are shown in Fig. 1. For last several years, extensive investigation was carried out to identify the active constituents and their mode of action in killing lungs and breast cancer cells.

Mode of action of malabaricones against breast cancer cells

There is a burgeoning interest in finding natural redox active molecules, which can often cleave DNA, and hence can be used as anti-cancer agents. Although, the redox potentials of a variety of metal ions have been exploited for the development of DNA cleaving agents, the organic compounds possibly play more important roles in this regard, as they provide a multitude of binding interactions with the target DNA, while ensuring the required electron transfer via their intrinsic chemical, electrochemical and photochemical properties [15]. Initially, we focused on assessing the potential of the malabaricones as the DNA cleaving agents, and whether this confers them with anti-cancer properties. The nuclease activities of the malabaricones (A–D) have been studied so as to establish a structure–activity correlation and deduce the mechanistic pathway of the process [15]. The inactivity of mal A and mal D revealed that the resorcinol moiety, present in the malabaricones did not contribute to the nuclease activity. Amongst the test compounds, mal C containing a B-ring catechol moiety showed significantly better Cu(II)-dependent nuclease activity than the partially methylated catechol derivative, mal B and curcumin (Fig. 2A). Mechanistically, mal C was found to bind efficiently with Cu(II) and DNA to facilitate the DNA nicking via a site-specifically generated Cu(II)-peroxo complex (Fig. 2B) [15]. Consistent with its Cu(II)-dependent nuclease property, mal C showed better cytotoxicity (IC50: 5.26 ± 1.2 μM) than curcumin (IC50: 24.46 ± 3.3 μM) against the MCF-7 human breast cancer cell line. The mal C-induced killing of the MCF-7 cells followed an apoptotic pathway involving oxidative damage to the cellular DNA (Fig. 2C) [15]. The cellular DNA fragmentation by mal C that involves mobilization of intra-cellular and extra-cellular Cu(II), could be one of the mechanisms involved in its chemopreventive property.

Further, our study revealed mal C induced mitochondrial damage. This was assessed by fluorescence microscopy and flow cytometric analyses of the JC-1-stained cells (Fig. 2 C&D) [16]. Besides, mal C treatment led to a significant increase in lysosomal membrane permeabilization (LMP), along with the release of cathepsin B, as well as
BID-cleavage and its translocation to mitochondria. Mal C induced LMP occurs prior to mitochondrial dysfunction in breast cancer cells. This suggested that cytotoxicity of mal C against human breast cancer cells may proceed through LMP as the initial event that triggered a caspase-independent, but cathepsin B and t-BID-dependent intrinsic mitochondrial apoptotic pathway.

Moreover, a significant accumulation of cells in the S or G2-M phases along with upregulation of the cyclins E and A due to mal C exposure promises it to be a potential anti-cancer agent [16].

Malabaricone C induced robust killing of lung carcinoma cells through a DNA damage dependent activation of CHK1-p38 MAPK pathway.

In order to evaluate the efficacy of malabaricones against lung carcinoma, the cytotoxic potential of malabaricones were tested against a panel of lung carcinoma (A549, NCI-H23, and NCI-H460) cells. Our results showed that mal A-D and curcumin induced cytotoxicity in A549 lung carcinoma, with IC50 values of 19.2 ± 4.2 μM (mal A), 8.4 ± 2.5 μM (mal B), 7.0 ± 1.8 μM (mal C), 20.3 ± 5.1 μM (mal D) and 41.7 ± 6.2 μM (curcumin). Further, the mal C also found to induce cytotoxicity with similar IC50 values in several other lung cancer cells e.g., IC50 of 7.7 ± 2.1 μM.
μM for NCI-H460, 9.9 ± 2.7 μM for NCI-H23, and 12.4 ± 3.4 μM for NCI-H522 cells [17].

Mechanistically, our detailed investigation showed that mal C mediates apoptosis in multiple lung cancer cells, which is primarily associated with its ability to induce DNA double strand breaks (DSBs) (Fig. 3A&B) [17]. Subsequently, DSBs cause ATM/ATR-mediated activation of CHK1. Further, CHK1 induced rapid phosphorylation of p38-MAPK, which paralleled with mitochondrial dysfunctions in terms of imbalance in BAX/BCL2 expression, cytochrome-C release [17]. This conclusion is based on the fact that mitochondria depleted and p38-MAPK inhibited lung cancer cells are resistant while BCL2 knockdown cancer cells show higher sensitivity to mal C treatment [17]. Together, mal C shows an impressive anti-cancer efficacy against human lung carcinoma cells.

**Augmentation of therapeutic potential of malabaricone C by using N-acetyl cysteine as thiol antioxidant**

Recently, targeting drugs to redox homeostasis of the cancer cells is considered as one of the key strategy for cancer therapy. Recent clinical studies showed that N-acetyl cysteine (NAC) treatment significantly decreased the metabolic heterogeneity and reduced Ki67 (a proliferation marker) with simultaneous enhancement in apoptosis of breast carcinoma in patients. However, it is not yet precisely known how thiol antioxidants enhance killing of cancer cells, or any putative agent can tweak the cancer cells survival program in NAC/glutathione (GSH)-assisted manner to make cancer cells vulnerable to death. To this end, we showed that a dietary compound, malabaricone C (mal C), generated copious amounts of reactive oxygen species (ROS) and also reduced the GSH level in lung cancer cells (Fig. 4A & B).

Paradoxically, although antioxidants supplementation reduced mal C-induced ROS, thiol-antioxidants (NAC/GSH) restored intracellular...
GSH level but enhanced DNA DSBs and apoptotic cell death induced by mal C (Fig. 4 B & C). Mechanistically, our results unraveled two tightly coupled biochemical mechanisms attributing this sensitization process by thiol antioxidants. Firstly, it was anticipated that during O$_2^\bullet$ radicals generation by mal C [25], its easily oxidizable catechol function will be converted to the corresponding ortho-quinone moiety (Fig. 5A). To this end, our results showed that the absorption spectra of mal C in culture media is shifted to progressive emergence of the quinone absorption peak at 480 nm, with simultaneous depletion of the mal C absorption at 373 nm (Fig. 5B). Co-incubation with NAC delayed the conversion, and the effect of NAC was concentration dependent. Moreover, the peak quinone levels in presence of NAC were much less vis-à-vis mal C incubation only (Fig. 6C). Together, our results showed that thiol antioxidants enable the “catechol-quinone redox cycle” of mal C and ameliorate ROS generation and bio-molecular damage (DNA and protein). Secondly, thiol antioxidants cause rapid glutathionylation of transcription factors [p53, p65 (NF-kB) etc.,] oxidized by mal C, and abrogates their nuclear sequestration.

Fig. 5. (A) Schematic representation for autoxdation of mal C and generation of quinone forms and ROS. (B) Incubation of mal C generates quinone form (480 nm) in a time dependent manner. (C) Oxidation of mal C to its quinone form was delayed and reduced by thiol antioxidants.

Fig. 6. Mal C treatment, in A549 lung carcinoma, enhances sequestration of p65 to nucleus. Mal C mediated nuclear sequestration of p65 protein was abrogated by NAC antioxidant.
and transcription of the anti-apoptotic proteins (Fig. 6). Furthermore, analyses of the mitochondrial fractions, of p53 expressing and silenced cells, revealed that cytoplasmic accumulation of glutathionylated p53 (p53-SSG) and p65 (p65-SSG) trigger a robust mitochondrial death process.

**Therapeutic efficacy of mal C alone and in combination with NAC in vivo tumor models**

Considering impressive anti-cancer potential of mal C alone and in combination with NAC in vitro, their efficacy was evaluated using a lung tumor xenograft mouse model. Tumor bearing mice were administered orally with vehicle e, mal C (100 mg/kg body weight), NAC or combination. As shown in Fig. 7 A-D, oral administration of mal C alone (100 mg/kg) resulted in significant reduction in both tumor volume and weight but the combination of mal C and NAC resulted in the most effective response in terms of tumor growth retardation compared to vehicle treated mice. In order to evaluate the anti-tumor efficacy against aggressive and nonresponsive tumors, we used B16F10 C57BL/6 murine melanoma tumor model. B16F10 melanoma tumors are aggressive, highly invasive and metastasizes to lungs. Interestingly, mal C dose (50 and 100 mg/kg body weight) dependently reduced both tumor volume and weight. Moreover, combination of mal C and NAC resulted further reduction in both these parameters. During treatment with mal C or NAC, either alone or in combination, there was no evidence of severe loss in body weight indicating that the combination was well tolerated. All these data support a potential use of mal C alone or in combination with NAC (thiol antioxidants) in the management of lung and melanoma tumors. Mal C is abundant in rampatri, which is extensively consumed in the eastern world without any side effect. Earlier, we found that mal C is non-toxic to mice, even at an appreciably high dose (500 mg/kg). Considering this encouraging findings, mal C appears to be a potent anti-cancer drug.

**Gram scale syntheses of malabaricones B and C**

Earlier, malabaricones, isolated from fruit rind of Myristica malabarica has been used for performing pharmacological studies in our laboratory. However, isolation of these compounds from plant extract has always remained tedious since the whole malabaricone family is chemically very closely related to each other. Besides, in vitro and in vivo evaluation of malabaricones for studying mechanism of action, pharmacological relevance and therapeutic efficacy warrants a requirement of significant amount of compound. To address this issue, we sought to develop a protocol for syntheses of malabaricones in gram scale.

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**Scheme 1. Total synthesis of malabaricone B and C via cross-metathesis strategy**
Olefin cross-metathesis has emerged as the most powerful catalytic carbon–carbon bond forming reactions leading to profound synthetic developments in the materials, agricultural, and pharmaceutical industries. This strategy has been successfully utilized in synthesis of ω-arylheptyl bromide (2), one of the key building blocks of our synthetic strategy (Scheme 1). Successful C-alkylation of 3, with 2 was achieved in presence of base and KI as an additive to obtain the β-ketoester (4). Compound 4 was then converted to mal B (1b) and mal C (1c) by means of alkaline hydrolysis, decarboxylation followed by demethylation (Scheme 1). The present protocol can be adopted for the synthesis of all other members of malabaricone family, including mal A (1a) and mal D (1d). Concise synthetic route using readily available inexpensive starting materials, high overall yield and simpler reaction conditions are some of the prominent features of our synthetic strategy. The cytotoxic properties of the synthesized mal B and mal C were found to be similar to those isolated from the natural resources.

**Library synthesis of malabaricones analogues for structure-activity relationship study towards the development of most potent cytotoxic agent**

Our studies revealed that mal C demonstrates anti-tumor properties with the potential for cancer treatment either alone or in combination with other agents, as observed in the breast, lung, colorectal, osteosarcoma and melanoma cancer. Besides, mal C shows higher therapeutic efficacy than curcumin and resveratrol against multiple cancer cells. In order to further ameliorate the therapeutic potential of mal C, we sought to understand the role of its different structural attributes in governing the cancer cell death process. We envisaged that the activity of mal C (Fig. 8) can be augmented by modifying its structure in the following ways such as (i) altering spacer length (ii) altering position and number of hydroxyl groups in its A and B ring (iii) removal of the B-ring and (iv) addition/removal of the carbonyl functionality. To this end, a series of malabaricone analogs (Fig. 8, ML-1 to ML-21) were synthesized by reacting different phenyl β-ketoesters and ω-aryl alkyl bromides/alkyl bromides by following our protocol used in synthesis of mal B and mal C. Evaluation of in vitro anti-cancer potentials of these analogues revealed...
that altering the functional groups in ring-A, -B, carbonyl group or change in aliphatic carbon chain length significantly reduced breast cancer killing potential of Mal C. However, amongst the analogues, the new tetramethoxy malabaricone (TMM, ML-6, Fig. 9) was more effective in inhibiting tumor cells growth when compared to Mal C. In clonogenic assay, the TMM showed IC50, which is 3 fold smaller than Mal C. Besides, TUNEL assay, Annexin-V staining, caspase activation and PARP cleavage analyses unveiled the ability of TMM to cause breast cancer death by triggering apoptosis. Further our detailed mechanistic evaluation revealed that TMM mediated higher cell death is associated with induction of DNA double strand breaks, abrogation of G2/M checkpoint and blockage of cell survival process i.e., autophagy (Fig. 9).

**Conclusions**

Natural products have always been an important source of bio-active compounds, and for the last several years, Bio-Organic Division, BARC, is deeply engaged in the development of phytochemicals as anti-cancer agents. To this end, an important polyphenolics viz. malabaricone C (Mal C), which was isolated from fruit rind of rampatri spice displayed impressive anti-cancer properties in vitro and in vivo tumor models. Mal C alone and in combination with NAC effectively reduced lung and melanoma tumor burden in preclinical mice models. Mechanistically, thiol antioxidants targets "catechol-quinone redox cycle" of Mal to generate copious amounts of ROS and cause higher DNA DSBs. Besides, thiol antioxidants cause glutathionylation of survival proteins, accumulation in cytoplasm and mitochondrial death process in response to Mal C treatment.

Keeping in view the involvement of laborious procedure for the extraction of malabaricones from plant matter as well as the requirement of larger amount of them for elucidation of their mechanism of action, an innovative protocol of their synthesis was earlier developed by us [18].

Furthermore, a library of malabaricone analogs was synthesized to evaluate the correlation of various functional groups with their biological activities which in turn will be useful in designing malabaricone congeners with improved cytotoxic efficacy. Gratifyingly, it was observed that tetrathylether of Mal C (TMM) (devoid of any phenolic OH group), the synthetic precursor of Mal C, and displayed better cytotoxicity against breast cancer than that obtained with Mal C.

This has prompted us to carry out detail investigation on the mechanism of their action, which is currently underway.

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