Chapter 13

Kuguacin J, a Triterpenoid from *Momordica charantia* Linn: A Comprehensive Review of Anticarcinogenic Properties

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1. Introduction

Momordica charantia (MC) L. belongs to a short-fruited group of the Cucurbitaceae family and has been widely cultivated as a vegetable crop in many tropical and subtropical countries. The fruit, vines, leaves and roots of this plant have been used as a traditional medicine for the treatment of toothaches, diarrhea, furuncle, and diabetes [1-3]. Its fruit, referred to as kugua in the Chinese language, Mara Kee Nok in Thai and bitter melon in English, has been used in Chinese, Indian and Thai Cooking. Bitter melon, also known as bitter gourd, is cylindrical shaped and 4 to 12 inches in length and 1 and a half to 3 inches in diameter and contains large seeds inside. It tastes very bitter and is considered a blood purifier. It can be cut into rings and deep-fried for a snack. It is also often stir fried with meat, shrimp or fish.

In view of the popularity of bitter melon in the Asian tropics and the fact that the results of using bitter melon as a remedy for diabetes has yielded conflicting results [2,4,5], more research needs to be done on its hypoglycemic activity. In addition, several compounds from bitter melon have shown interesting pharmacological activities, including antitumor, immunotoxic and anti-HIV properties, which merit further research, and may have strong potential in the development of future medicines [2,6-9]. Although different types of synthetic drugs are available for the treatment of chronic diseases, such as diabetes and cancer, the synthetic agents in use can produce serious side effects and toxicity. Hence there is a demand for safer and more effective agents. In many parts of the world, an Ayurvedic (traditional Indian) approach has been used for the treatment of a number of diseases, including diabetes and cancer and therein exists a hidden wealth of potential for useful natural products in the control of diseases. The World Health Organization (WHO) has

recommended that this area warrants further evaluation that might reveal effective dietary adjuncts, either for the treatment or prevention of specific diseases [10]. With traditional use supported by modern scientific evidence of the beneficial function of MC, it is one of the most promising plants for drug development. However, its phytochemicals and mechanism of action are poorly understood. Extensive research has studied the isolation of several classes of active components, including cucurbitane-type triterpenoids and the potential biological and pharmacological activities of natural products in MC [7,11]. The aim of this chapter is to review the cucurbitane type triterpenoids, particularly kuguacin J, in MC with the goal of encouraging future studies.

1.1. Geographic distribution and monograph

MC is an herbaceous, tendril-bearing vine growing up to 5 m in length. It bears simple, alternate leaves, with 3-7 deeply separated lobes. Each plant bears separate yellow male and female flowers [12]. Fruit 2.5-25 cm long, oblong, pendulous, fusiform, usually pointed or beaked, ribbed and bearing numerous triangular tubercles, 3 valves are present at the apex when mature. MC are perennial climbers cultivated throughout India and in Southern China and is now found naturalized in almost all tropical and subtropical regions. It is an important vegetable in India, Sri Lanka, Vietnam, Thailand, Malaysia, the Philippines and Southern China. It is also cultivated on a small scale in tropical America. MC is grown mainly for the production of immature fruit, although the young leaves are edible as a vegetable [3]. The young fruit is emerald green, but turns to orange-yellow when ripe. At maturity, the fruit splits into irregular valves that curl backwards and release numerous brown or white seeds encased in scarlet arils (Figure 1). All parts of the plant, including the fruit, taste bitter. It has been used extensively in traditional medicine as a remedy for diabetes.

Figure 1. Flowers, immature fruit and young leaves of *Momordica charantia* (MC) at a home garden in Chiang Mai, Thailand (the picture was taken in March, 2012).

1.2. Pharmacological activities

Previous investigations have shown that water extracts of the leaf and fruit of MC exhibited high antioxidant activity and that bitter melon fractions are rich in phenolics and have a

strong antioxidant activity [13]. The role of free radicals and active oxygen in treating chronic diseases including cancer, aging and atherosclerosis has been recognized [14]. Therefore, much attention has been focused on the use of antioxidants in protecting against the threat of damage of free radicals. The active fractions from the fruit of MC have been reported to have significant hypoglycemic and antidiabetic effects [1,5]. Its active compounds were reported to be saponins [15,16] and peptides [17,18]. Charantin, an antidiabetic compound, is a typical cucurbitane-type triterpenoid in MC and is a potential and promising substance for the treatment of diabetes [19]. A number of studies have reported the effects of MC unrelated to diabetes. MC has some interesting biological and pharmacological activities. Extracts of MC have been reported to possess antitumor activity, such as the inhibition of mouse spontaneous mammary tumourigenesis [20] and benzo(a)pyrene-induced mouse forestomach tumourigenesis [21]. Besides, antioxidant activities [22], the antiviral [9], antidiabetic and immunomodulating properties [23] of this plant have also been explored.

1.3. Ethnomedical uses

The fruit, leaves and roots of MC have been used in Ayurveda in the treatment of a number of diseases. They have been used as a bitter stomachic, a laxative and an anthelmintic [24]. The whole extract of the fruit is also advocated in the treatment of diseases of the spleen, liver, and in rheumatism and gout [25]. An active ingredient from this plant has been used in diabetes mellitus [6,11,26]. In KwaZulu-Natal, the Zulus drink a concoction of the root or leaf for the treatment of boils and take an infusion of the runner as a sedative for an irritable stomach [27]. It has also been reported that it has been used as a remedy for hypertension and is reported to have antidiabetic properties. The leaf is used by the Chagga as an earache remedy [28] and in tropical Africa, the leaf is used to treat roundworm [29]. In Uganda, an infusion of the leaf and roots is used as an abortifacient and ecbolic [28] and in Tanzania the fruit pulp is regarded as being poisonous to weevils, moths and ants and is used as a repellant [30]. In the Philippines, the leaves are often used for children's coughs. It is used in the treatment of skin diseases, sterility in women, as a parasiticide, as an antipyretic, and as a purgative. MC is also known as the Ampalaya tree, which is a vegetable grown throughout the Phillipines [31] and Ampalaya tea is a bitter brew made from the fruit or leaves. Ayurveda knowledge has claimed that Amapalaya stimulates digestion, helping those with dyspepsia or constipation. The tea seems to help people with diabetes mellitus control the disease, and it may provide some antimalarial benefits. Ampalaya has been traditionally regarded by Asians, as well as Panamanians and Columbians, as being useful in the prevention and treatment of malaria. Laboratory studies have confirmed that various species of the bitter fruit have anti-malarial activity, although human studies have not yet been published. In the Philippines, the effect of MC capsule preparation on glycemic control in type 2 diabetes mellitus has been studied [32]. However, there was no significant effect on mean fasting blood sugar, total cholesterol, and weight or on serum creatinine, ALT, AST, sodium and potassium in 40 outpatients who received treatment at the Phillippine General Hospital.

1.4. Phytochemicals and cucurbitane triterpenoids

Since the early 1960's the constituents of bitter melon have been investigated and several classes of primary and secondary metabolites have been isolated from MC fruits, seeds and whole plants. It contains biologically active chemicals that include crude fat, crude protein, soluble dietary fiber, minerals, essential oil, flavonoids, phenolic acids, glycosides and triterpenes. The young fruit is a good source of vitamin C and vitamin A [33-35]. A steroid saponin called charantin has been isolated from the fruits and leaves. It also contains a polypeptide named gurmarin, which is similar to insulin in composition.

Among the secondary metabolites of MC, cucurbitane-type triterpinoids are one of the main bioactive constituents. The terpenoids, refererred to as isoprenoids, are a class of natural products and the related compounds have been formally derived from five-carbon isoprene units. Terpenoids of different sizes and composition are found in all classes of living things and are the largest groups of naturally occurring chemicals. This class has been subdivided according to the number of carbon atoms. The triterpenoids are terpenoids with a C30 skeleton. These C30 constituents are isolated and characterized from various sources in nature, particularly in resins and may occur as either esters or glycosides [36,37]. More extensive backbone rearrangement of the protostane cation affords the curcubitane skeleton. The cucurbitacins are a typical group of cucurbitane-type triterpenoids found in plants and belong to the cucumber family (Cucurbitaceae). The natural cucurbitracins are well-known for their bitterness and toxicity [38]. The cucurbitane-type triterpenoids and their aglycones have shown some biological effects beneficial in treating diabetes and obesity, and possess anticancer, anti-HIV and antifeedant properties [7,39]. More than fifty cucurbitacins and cucurbitane glycosides from the fruits, seeds, leaves, vines and stems have been reported [40-48]. Recently, Chen et al (2008, 2009) isolated and structured elucidation of nineteen cucurbitacins named kuguacins A-E from the roots of MC [49] and kuguacins F-S [50] from the vines and leaves of MC. They claimed that some of them are artifacts formed during the extraction process. The kuguacin C and E showed moderate anti-HIV-1 activity with EC_{50} values of 8.45 and 25.62 μg/ml. The kuguacins F-S exhibited weak anti-HIV activities *in vitro*.

Recently, several triterpenoids from various plants have been reported to feature anti-proliferative [51-53] and anti-invasive [54,55] bioactive components. More than 50 triterpenoids have been isolated from bitter melon, but their biological activities have yet to be explored in detail. However, it has been shown that Cucurbitacin B (cucB), a triterpenoid from Cucurbitaceae vegetables also found in bitter melon seeds, has caused cell cycle arrest and apoptosis induction in human colon adenocarcinoma cancer cells [53]. Moreover, 2 flavonoids and 4 phenolic acids, including rutin, naringin, gentistic acid, benzoic acid, *o*-coumaric acid, and *t*-cinnamic acid, are present in bitter melon leaves [35]. Rutin, a flavonoid glycoside, has been reported to successfully show growth inhibition of leukemia and ovarian carcinomas, with anti-invasive effects on melanoma [35,56-58]. Triterpenoids and flavoniods included in bitter melon leaf extract (BMLE) might be promising components with critical roles against cancer cell progression, but the active compound(s) remain to be identified.

This review presents previously published and current information regarding a cucurbitane type triterpenoid, kuguacin J, and provides new insights into the underlying potential of the chemical isolation and biological activities of kuguacin J in MC. Molecular mechanisms of kuguacin J, as a promising anticancer agent, will be discussed in detail.

1.5. Isolation and purification of kuguacin J from MC leaf

Our previous study [59] has compared the effects of the ethanolic extracts from leaves, fruits and tendrils of MC on drug accumulation and P-gp activity *in vitro*. The leaf extract (BMLE) showed a concentration-dependent effect on P-gp-mediated vinblastine accumulation and effux in drug resistant KB-V1 cells, but had no effect in drug-sensitive KB-3-1 cells, which lack P-gp, while there was no change in drug accumulation and efflux in KB-V1 cells in the presence of the fruit and tendril extracts [59]. These firstly inspired us to further identify the active component(s) of BMLE, which modulate the function of P-gp and the multidrug-resistant (MDR) phenotype in multidrug-resistant human cervical carcinoma KB-V1 cells using Bioassay-guided fractionation.

In the next study [60], the ethanolic fraction, BMLE, was subsequently extracted with solvents increasing in polarity (i.e., ethanol, hexane, diethyl ether, chloroform and ethyl acetate). These extraction samples were tested for their abilities to modulate the function of P-gp in the multidrug-resistant human cervical carcinoma KB-V1 cells in comparison with wild type drug sensitive KB-3-1 cells. Among the extracts tested, the hexane and diethyl ether fractions had the most effective MDR reversing properties, and increased intracellular [^{3}H]-vinblastine accumulation and decreased the [^{3}H]-vinblastine efflux in KB-V1 cells. The percent yield of the diethyl ether fraction was higher than the hexane fraction. Moreover, in another of our studies, the growth inhibitory effects on LNCaP cells showed that the diethyl ether fraction (DEF) of BMLE displayed the strongest inhibitory effect on the cell growth [61]. So, we therefore purified the active component using the diethyl ether fraction as a principle material. Bioassay-guided fractionation led us to isolate a purified white crystal. The IR, NMR and MS data of the compound was compared with previous reports [62] and it was identified as kuguacin J (3,7,23-trihydroxycucurbita-5,24-dien-19-al), (Figure 2). We further characterized the anticancer properties of BMLE (*in vitro* and *in vivo*) and kuguacin J (*in vitro*) and these will be further discussed in the following sections.

2. Bitter melon and cancer

Cancer is a disease in which the cell presents itself with uncontrolled proliferative potential. The transition of normal cells towards the cancerous phenotype occurs at various stages [63]. Since these defects are mostly due to aberrant signaling cascades involving numerous molecular players, targeting them by chemopreventive agents at any stage could be a rationalized approach in achieving the control of cancer.

Carcinogenesis is generally a complex and multi-step process in which distinct molecular and cellular alterations occur. In order to simplify the understanding of the different

possible options for chemoprevention and chemotherapy in cancer development and progression, the following stages have been described: (i) initiation, when cells are exposed to a carcinogenic agent, (ii) promotion, when abnormal cells persist and initiate a preneoplastic stage (iii) progression, final phase of the tumorigenesis, when uncontrolled cell growth is resistant to anticancer drugs (Multidrug Resistance; MDR), and aggressiveness or metastasis occur. A cancer chemopreventive agent could be effective at any of the classically defined stages of carcinogenesis: initiation, promotion, and/or progression [64,65]. As discussed earlier, extracts of bitter melon have been reported to possess anti-tumor activity [20,21] and the following sections detail the signaling cascades that are targeted.

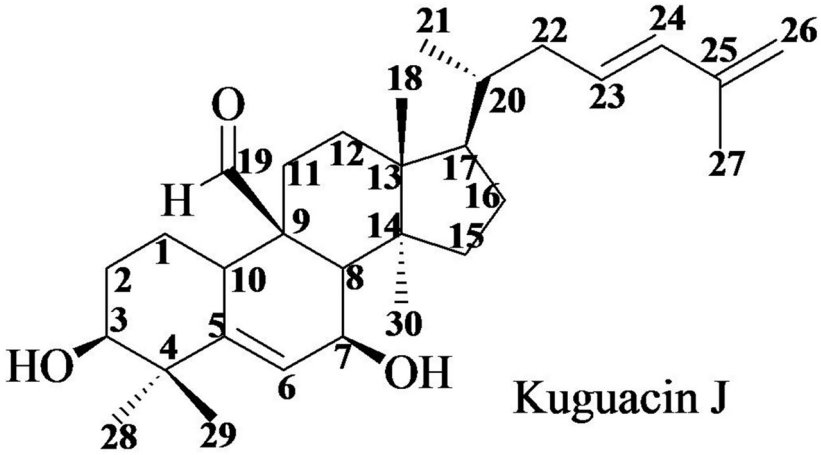

Figure 2. Chemical structure of kuguacin J isolated from MC.

2.1. Anticancer *in vitro* studies

Prevention by the use of naturally occurring dietary substances is considered a practical approach in reducing the increasing incidence of cancer. The intervention of multi-stage carcinogenesis by modulating intracellular signaling pathways may provide the molecular basis of chemoprevention with a wide variety of dietary phytochemicals [66,67].

Anticancer activities of MC against numerous cancers have revealed that it contains active compounds that provide anticancer potential to inhibit cell growth and proliferation, as well as to induce apoptotic cell death in several cancer cell lines [68-71]. Our studies [60,61,72,73] found that kuguacin J, one of the active compounds contained in MC extracts, exerts anticancer properties in various experimental models, which will be discussed further in the following sections.

2.1.1. Antiproliferative and antiapoptotic activities

Generally, deregulation of cell growth and resistance to apoptosis are the major defects in uncontrolled cancer cell growth, hinting that development of approaches that induce cell

cycle arrest and apoptotic machineries in cancer cells could be effective measures against their proliferation. Recently, dietary phytochemicals are considered promising chemopreventive or chemotherapeutic agents [67,74]. Thus, the induction of cell cycle arrest or apoptosis using dietary chemopreventive compounds might be an excellent approach to inhibit the promotion and progression of carcinogenesis and to remove genetically deregulated, premalignant and malignant cells from the body. We have investigated the growth inhibitory effect of BMLE and kuguacin J on androgen-dependent and androgen-independent prostate cancer models [61,72,73].

Our study [61] found that BMLE exerted significant growth inhibitory effects on LNCaP via the induction of G1 arrest and apoptosis cell death and the alteration of cyclin D1, PCNA, Bcl-2/Bax, caspase-3 and cleaved caspase-3 protein levels. The data prompted us to characterize the active compound(s) in the extract, which could be valuable in the prevention and intervention of cancers. It was found that kuguacin J exerted a marked decrease of LNCaP cell proliferation and viability, suggesting that kuguacin J is at least one of the active components in BMLE that plays a critical role in the growth inhibition of LNCaP.

The treatment of androgen-sensitive (LNCaP) cells with kuguacin J resulted in a significant G1-phase arrest of cell cycle progression [61], which indicates that one of the mechanisms by which kuguacin J may act to inhibit the proliferation of cancer cells is the regulation of cell cycle progression. We next examined the effect of kuguacin J on cell cycle regulatory molecules operative in the G1 phase of the cell cycle. The reduction of cyclinD1, cyclinE, Cdk2 and Cdk4 in LNCaP cells by kuguacin J suggests the disruption of the uncontrolled cell cycle progression of these cells and that the kuguacin J-induced G1 arrest is mediated through the up-regulation of p21 and p27 proteins, which enhances the formation of heterotrimeric complexes with the G1-S Cdks and cyclins, thereby inhibiting their activity. Additionally, kuguacin J also dramatically suppressed the expression of a proliferation marker, PCNA, which is expressed late in the G1 phase and early in the S phase [75]. The inhibition of cell proliferation or the induction of cell death in LNCaP by kuguacin J might be associated with the G1 arrest machinery.

G1-phase arrest of cell cycle progression provides an opportunity for cells to either undergo repair mechanisms or follow the apoptotic pathway. Apoptosis plays a crucial role in that it functions as an essential mechanism of tissue homeostasis, eliminating the mutated neoplastic and hyperproliferating neoplastic cells from the system. Acquired resistance toward apoptosis is a hallmark of most, and perhaps all, types of cancer. Cancer cells become resistant to apoptosis and do not respond to the cytotoxic effects of the chemotherapeutic agents [76]. Therefore induction of apoptosis is considered a protective mechanism against cancer progression. We thus determined the effect of kuguacin J on the induction of apoptosis in LNCaP cells [61]. Kuguacin J treatment caused significant induction of apoptosis. Hence, kuguacin J seems to be a potent chemotherapeutic agent for prostate cancer inhibition. Moreover, kuguacin J treatment reduced the protein level of surviving, which might be associated with kuguacin J-induced cell cycle arrest and apoptosis in LNCaP. Kuguacin J treatment could alter the protein levels of key members of

the Bcl-2 family in a manner that favors an increase in the ratio of Bax/Bcl-2 and Bad/Bcl-xL and increases the activation of caspase-3 and cleavage of PARP. This may represent the mechanism by which cancer cells are susceptibility to kuguacin J mediated apoptosis.

LNCaP is an androgen-dependent, androgen receptor (AR)-positive prostate cancer cell line. AR is known to play a critical role in the development and progression of prostate cancer [77]. The ability of AR to cross-talk with several growth factor signaling cascades toward the regulation of cell cycle, apoptosis, and differentiation outcomes in prostate cancer cells has been reported [77]. Inhibition of AR activity could be the major therapeutic goal to delay prostate cancer progression. Kuguacin J decreased the expression of AR followed by the reduction of the protein level of PSA [61], which is one major AR-dependent target gene. The diminishment of PSA represents the effective inhibition of AR activity by kuguacin J treatments. The decrease of AR might be involved in the kuguacin J-caused growth inhibition of LNCaP through the induction of G1 arrest and apoptosis.

The tumor suppressor p53 protein is a regulator of genotoxic stress that plays an important role in DNA damage response, DNA repair, cell cycle regulation, and in triggering apoptosis after cell injury [78]. Induction of apoptosis is considered to be central to the tumor-suppressive function of p53 [79]. Kuguacin J-treated LNCaP cells markedly increased the expression of the p53 protein [61]. We further investigated whether kuguacin J-induced cell cycle arrest and apoptosis of LNCaP cells are p53-dependent using p53 RNA interference. We found that kuguacin J induced p53-mediated partly cell cycle arrest, and mainly apoptosis, which led to the inhibition of cell growth and may be related to the activation of p53 signaling pathway [61].

Treatment of androgen-independent prostate cancer cells (PC3) with kuguacin J caused a significant G1-phase arrest along with a reduction of survivin, cyclinD1, cyclinE, Cdk2 and Cdk4 [73]. Kuguacin J also dramatically suppressed the expression of a proliferation marker, PCNA, that is expressed in both late G1 phase and early S phase [61,73] .

Kuguacin J appears to inhibit the growth of the androgen-dependent LNCaP cells to a greater degree compared to the androgen-independent PC3 cell line [61,73]. A number of differences between PC3 and LNCaP cells may account for this difference in sensitivity. PC3 is a more aggressively growing cell line and is null for both p53 and the AR. LNCaP is a much less aggressive cell line and possesses wild-type p53 and an AR, which although mutated, is responsive to androgen. Our study revealed that kuguacin J markedly decreased AR expression and induced p53 levels in LNCaP cells. We also found that p53 played a critical role in the kuguacin J-mediated induction of apoptosis by LNCaP cells. The data could explain the lower induction of apoptosis in PC3 cells compared to LNCaP. The induction of G1 arrest by kuguacin J, however, was similar in AR-dependent LNCaP cells and AR-independent PC3 cells, suggesting that the AR might not be a critical component in mediating the growth-arresting properties of kuguacin J.

The sensitivity of the human normal prostatic epithelial cell line PNT1A to the cytotoxic effects of kuguacin J was much lower than that of the prostate cancer cell lines (LNCaP and PC3) [61]. Thus, kuguacin J could be an effective chemopreventive and chemotherapeutic

agent against prostate cancer, which might have no or a low side effect on normal cells and tissues.

Our studies [61,73] provide the evidence that shows the significant inhibitory effects on carcinogenic progression of LNCaP and PC3 cells via inhibition of cell growth and proliferation by kuguacin J. Thus, kuguacin J is able to induce apoptosis/cell cycle arrest in pre-initiated/initiated tumor cells, while in more advanced tumors kuguacin J is still able to induce apoptosis/cell cycle arrest. Taken together, kuguacin J might be a promising candidate as a new chemopreventive agent for both androgen-dependent and androgen-independent prostate cancer and could be developed as an alternative treatment option in cancer therapy. The *in vivo* study and growth inhibitory effects of kuguacin J on other cancers has to be further investigated.

Because kuguacin J is a purified compound from BMLE, the differences of the anti-tumor effects between kuguacin J and BMLE were investigated. The growth inhibition effects of kuguacin J on LNCaP are similar to BMLE with the phenomena of cell cycle arrest and apoptosis induction and alteration of the expression of cell cycle- and apoptosis-regulators. However, kuguacin J is included as only 1.6% of the BMLE, and the effective concentration to inhibit cancer cell growth of kuguacin J was 10 times lower than that of BMLE [60,61,73]. Therefore, BMLE may include other compounds, which also have anti-tumor function towards LNCaP cells.

2.1.2. Multidrug resistance reversing properties via modulation of P-glycoprotein function

The development and strategic use of anticancer drugs has become one of the most important ways of controlling malignant diseases. However, the emergence of drug resistance has made many of the currently available chemotherapeutic agents ineffective. Efforts to reverse the drug resistance of tumor cells have been largely unsuccessful [80]. In recent years, considerable research has been directed toward understanding the underlying mechanisms that confer drug resistance. Many studies using tumor cell lines as model systems have demonstrated that the exposure of cells to one drug often results in cross-resistance to many other structurally, chemically, and functionally distinct agents. This phenomenon is broadly known as the MDR phenotype [81-84]. The mechanism of MDR has now shown that some of the ATP-binding cassette (ABC) transporter proteins, especially ABCB1, or as it is more commonly referred to in literature, P-glycoprotein (P-gp), which is normally expressed in tumors derived from epithelial tissues including cancers of the kidney, liver, colon, and brain, has been associated with the intrinsic drug resistance of these cancers [85]. Some other tumors (for example breast, ovarian and small cell lung cancers) exhibit generally low levels of P-gp expression at diagnosis. However, the P-gp expression can be induced during the course of treatment, causing the cancer to become resistant to anticancer drugs [85]. At present, due in part to the disappointing results associated with the many side effects of P-gp modulators that have been used in clinical trials, current research efforts have been directed towards the identification of novel compounds with an attention toward dietary natural products or dietary herbs. The advantage is that these dietary herbs

exhibit little or virtually no side effects and do not further increase the patient's medication burden.

Our study investigated the effects of bitter melon extracts from leaves, fruits and tendrils on drug accumulation and P-gp activity *in vitro* [59]. We found that the leaf extract showed the greatest efficacy on P-gp-mediated vinblastine accumulation and effux in drug resistant KB-V1 cells, but had no effect in drug-sensitive KB-3-1 cells, which lack P-gp. There was no change in drug accumulation and effux in KB-V1 cells in the presence of fruit and tendril extracts. The protein expression level of P-gp in KB-V1 cells was not altered by BMLE. Therefore, BMLE possibly modulates intracellular drug levels by inhibiting P-gp activity. We next identified the active component(s) in BMLE that act to modulate the function of P-gp and the MDR phenotype in multidrug-resistant human cervical carcinoma using Bioassay-guided fractionation and led us to isolate a purified white crystal that was further identified as kuguacin J [60].

Kuguacin J increased the sensitivity of KB-V1 cells to vinblastine and paclitaxel, but did not have this effect on KB-3-1 cells [60]. Moreover, the treatment of KB-V1 and KB-3-1 cells with kuguacin J yielded a marked increase in C-AM and Rh123 accumulation in a concentration-dependent manner in KB-V1 cells, but had no effect on KB-3-1 cells. C-AM and Rh123 are known to be good substrates for P-gp, indicating that kuguacin J modulates intracellular drug levels by inhibiting P-gp activity. The inhibitory effect of kuguacin J on P-gp function by [^{3}H]-vinblastine transportation assays showed that kuguacin J also increased [^{3}H]-vinblastine accumulation in KB-V1 cells and decreased [^{3}H]-vinblastine efflux from KB-V1 cells. As our previous study showed, P-gp expression in KB-V1 cells was not affected by treating the cells with BMLE [59]. Our data suggests that kuguacin J inhibits P-gp activity, but does not inhibit its expression.

The photoaffinity labeled transport substrate of P-gp, [^{125}I]-IAAP, has been used extensively to study the interaction of the modulators at the substrate-binding site of P-gp [86]. It is also known that P-gp can be specifically labeled with [^{125}I]-IAAP, and the drug-substrates of P-gp can inhibit the photocrosslinking of [^{125}I]-IAAP to P-gp [87]. Kuguacin J inhibited the photocrosslinking of [^{125}I]-IAAP to P-gp in a concentration-dependent manner. This is direct evidence that kuguacin J interacts with the substrate-binding site of P-gp. We further verified the interaction of kuguacin J with P-gp using an ATPase assay, which is another useful assay in the study of the interaction of transport-substrates with P-gp, as ATP hydrolysis is coupled with the transport function of this transporter [88]. Although kuguacin J had a negligible effect on the basal ATPase activity of P-gp, a low concentration of kuguacin J slightly stimulated P-gp-mediated ATP hydrolysis. This finding also implies that kuguacin J interacts with the substrate-binding site of P-gp. We next investigated the effect of kuguacin-J on verapamil-stimulated ATPase activity to further characterize the nature of the interactions of kuguacin J. Kuguacin J indeed inhibited the verapamil-stimulated ATPase activity of P-gp. Furthermore, the kinetic analyses clearly showed that kuguacin J competes with verapamil for the substrate-binding site of P-gp. Taken together, the biochemical data suggest that kuguacin J inhibits the transport function of P-gp by interacting with the substrate-binding site of P-gp where verapamil also binds.

These results demonstrate that kuguacin J, an active compound isolated from BMLE, is an effective inhibitor of P-gp activity, and could be a candidate molecule for treating cancers exhibiting P-gp-mediated MDR. Investigations in animal experiments to determine whether kuguacin J has potential as an effective chemosensitizer that could be used in combination with conventional chemotherapy, needs to be explored.

2.1.3. Modulation potential on cancer cell metastasis

Metastasis, the spreading of malignant cells from the primary site to form a tumor mass at distant organs of the body, is the major cause of death in cancer patients. One of the critical characteristics of a metastatic cell is its invasion or ability to penetrate and invade the extracellular matrix and surrounding tissue. Invasion itself is a multistep process, requiring the coordination of various events, including the alteration of cell adhesion, promotion of cell migration, and degradation of the extracellular matrix barrier. Specifically, metastatic cells adhere to the basement membrane, secrete matrix-degrading enzymes such as matrix metalloproteinases (MMPs) to degrade the extracellular matrix barrier, and migrate from its original site. Therefore, the inhibition of these steps might be an effective approach in the prevention of cancer metastasis. The agents used in cancer metastasis therapy have been cytotoxic, with serious side effects that can diminish the quality of life of the cancer patients [89]. Recently, many efforts have therefore been made to search for non- or low-cytotoxic agents, which can reduce the spread of malignant tumors. One focus is to target cell invasion using substances found in medicinal plants [90,91].

We have provided clear evidence that BMLE can exert inhibitory potential against the metastatic properties of PLS10 cells *in vitro* [72]. Our results pointed to the beneficial effects at non-toxic levels. BMLE might thus afford an advantageous anti-cancer progression agent especially for tumor metastasis therapy. Non-cytotoxic BMLE treatment dramatically reduced migration and invasion properties and reduced, not only secretion, but also expression of MMP-2 and MMP-9 in androgen-independent rat prostate cancer cells. Additionally, uPA, which is an upstream enzyme of MMPs also implicated in tumor cell invasion, survival, and metastasis [92,93], was similarly reduced by BMLE treatment. Since TIMPs have the ability to form tight 1:1 complexes with the active MMP enzymes, changes in TIMP levels directly affect MMP activity [94,95]. BMLE treatment induced the expression of TIMP-2 that may be involved in the inhibition of tumor cell invasion. In addition, BMLE slightly reduced the proteolytic activity of collagenase type IV. Therefore, BMLE might mainly reduce the activity of MMPs by suppression of u-PA and induction of TIMP-2, and also may partially inhibit MMP activity through direct action.

The exact nature of the active components of BMLE, which exerts anti-invasion effects, now needs to be explored along with further elucidation of the underlying molecular mechanisms. Kuguacin J, which already has been investigated for its effects to inhibit P-gp function and reverse MDR in cervical carcinoma [60] and to induce G1 arrest and apoptosis in human prostate cancer cell lines [61,73], could be a candidate for a purified compound with inhibitory activities against cancer cell invasion and metastasis.

Our study showed that non-cytotoxic levels of kuguacin J dramatically reduced the migration and invasion of androgen-independent human prostate cancer PC3 cells [73]. Kuguacin J treatment reduced the secretion of active MMP-2, but did not reduce mRNA expression. Importantly, kuguacin J treatment also reduced the expression of MT1-MMP. Besides, kuguacin J inhibited the secretion of active MMP-9 and uPA. MMP-9 expression also appeared to be reduced by kuguacin J, but the reduction was not significant. The activity of purified collagenase type IV was not directly modulated by kuguacin J, indicating that kuguacin J-mediated the inhibition of the PC3 MMP enzymes, but was not affected by the direct inhibition of their collagenase activities.

BMLE and kuguacin J exert inhibitory effects *in vitro* on the progression of androgen-independent rat and human prostate cancer cells, respectively, by suppressing cancer cell invasion and metastasis [72,73]. These provide a basis for the use of BMLE as a dietary supplement and kuguacin J as a broader antineoplastic agent for cancer progression. Further studies are underway to explore the molecular mechanisms of the action of kuguacin J and to determine its properties *in vivo*.

2.2. Anticancer *in vivo* studies

Kuguacin J has only been recently isolated [62], thus the chemopreventive effects of kuguacin J on cancer or the study of carcinogenesis *in vivo* have not been elucidated yet. An important limitation is that the yield of kuguacin J obtained after purification was not enough to perform *in vivo* experiments. On the other hand, bitter melon extract (BME), from which kuguacin J was purified, was reported to have anticancer effects in *in vivo* studies. The bitter melon seed or fruit extracts were shown to have anticancer activities in a rat colonic aberrant crypt foci model [96] and a mouse mammary tumor model [20]. Extracts of bitter melon fruits were reported to inhibit tumor formation of lymphoma cells (L1210 and P388) with intraperitoneal injections of BME inhibited tumor formation in CBA/H mice [8]. BME also inhibited aberrant crypt foci, which is known as preneoplastic lesion, in the rat colon [97]. In addition, seed oil from bitter melon was reported to inhibit aberrant crypt foci and carcinogenesis induced by azoxymethane in the rat colon through an elevation of peroxisome proliferator-activated receptor gamma expression and an alteration of lipid composition [98].

Other purified materials from BME were also reported to inhibit cancer and/or carcinogenesis. Momordica protein of 30 kDa (MAP30) from MC was reported to inhibit breast cancer in the tumor xenograft model. The treatment of human breast cancer-bearing SCID mice with MAP30 resulted in significant increases in survival, with 20–25% of the mice remaining tumor free for 96 days [99]. Administration of MCP30 decreased PC3 human prostate cancer cell growth by the induction of apoptosis in nude mice [71]. Recent studies further indicate that the chemical modification and reduction of ribosome-inactivating protein in BME, significantly reduced its *in vivo* immunogenicity, but retained its anti-proliferative activity as measured by DNA fragmentation and caspase-3 activation [100]. Cucurbitane-type triterpenoids, charantosides, from a methanol extract of the fruits of MC

also inhibited mouse skin carcinogenesis induced by 7,12-dimethylbenz[a]anthracene (DMBA) or peroxynitrite plus 12-O-tetradecanoylphorbol-13-acetate (TPA) [45] α-ESA in bitter melon seed oil suppressing the growth of DLD-1 human colon cancer cells by apoptosis induction via lipid peroxidation [101]. α-ESA, which is converted to conjugated linoleic acid *in vivo*, had a stronger suppressive effect than the conjugated linoleic acid on tumor cell growth.

We also have reported the anti cancer abilities of BMLE *in vivo* [72,73]. Dietary BMLE treatment tended to reduce lung weight and the number of lung metastatic tumors in a model in which intravenous inoculation of androgen-independent rat prostate cancer cells was injected into nude mice, resulting in a 100% incidence of lung metastasis [72]. Treatment of BMLE significantly reduced the percentage of the tumor area in the lungs in a dose-response manner.

In another study, while kuguacin J inhibited the migration and invasion of PC3 cells *in vitro*, dietary BMLE did not reduce metastasis to the lymph node but significantly reduced the growth of PC3 xenografts [73]. One reason why the differences in the incidence of lymph node metastasis between the control and the BMLE-diet fed animals could not be determined is that the PC3 xenograph model had a very low incidence of metastasis. Taking all the mice together, PC3 cells metastasized to the lymph node with an incidence of only 3/20, 15%, while metastasis to other organs was not detected.

Our *in vitro* data presented that the mechanism of the anticancer effects of kuguacin J were similar to BMLE in androgen-dependent and -independent prostate cancer via cell cycle arrest, apoptosis and invasion [61,72,73], suggesting that the possibility of *in vivo* anticancer effects of kuguacin J might be similar to BMLE. Therefore, further work using suitable *in vivo* models will be necessary to fully understand the *in vivo* activity of kuguacin J.

With respect to *in vivo* toxicity, MC was shown to be safe with no signs of nephrotoxicity and hepatotoxicity without any adverse influence on food intake, growth organ weights and hematological parameters in experimental animals when ingested in low doses up to a period of 2 months [4,102]. The relatively low toxicity of all parts of this plant has also been reported when ingested, while toxicity and even death in laboratory animals has only been reported when the extracts were administered intravenously or by intraperitoneal injection in high doses [103]. MC has shown abortifacient activity traditionally, as well as experimentally [3,7]. The fruit and seeds demonstrated greater toxicity than the leaf or aerial parts of the plant. The documented adverse effects of MC are hypoglycemic coma and convulsions in children, reduced fertility in mice, a favism-like syndrome, increases in gamma-glutamyltransferase and alkaline phosphatase levels in animals, as well as headaches [3].

3. Conclusion

A current strategy for the evaluation of anticancer phytochemicals is based in part on: (1) cell cycle and apoptosis regulation; (2) anti-oxidative stress and anti-inflammatory activities;

(3) drug resistance of cancer cells; and (4) specific molecular targets targeting carcinogenesis and metastasis. In our studies, BMLE and one of the tritepenoids included in BMLE, kuguacin J, have shown that they possess potent anticancer capabilities to induce apoptosis/cell cycle arrest in pre-initiated/initiated tumor cells, while in more advanced tumors, these compounds could block resistance to anticancer drugs, tumor progression and metastasis (Figure 3). These findings provide evidence of the anticancer effects of BMLE and kuguacin J (as summarized in Figure 3) and suggest the strong possibility that these natural products can be developed for cancer chemoprevention and chemotherapy.

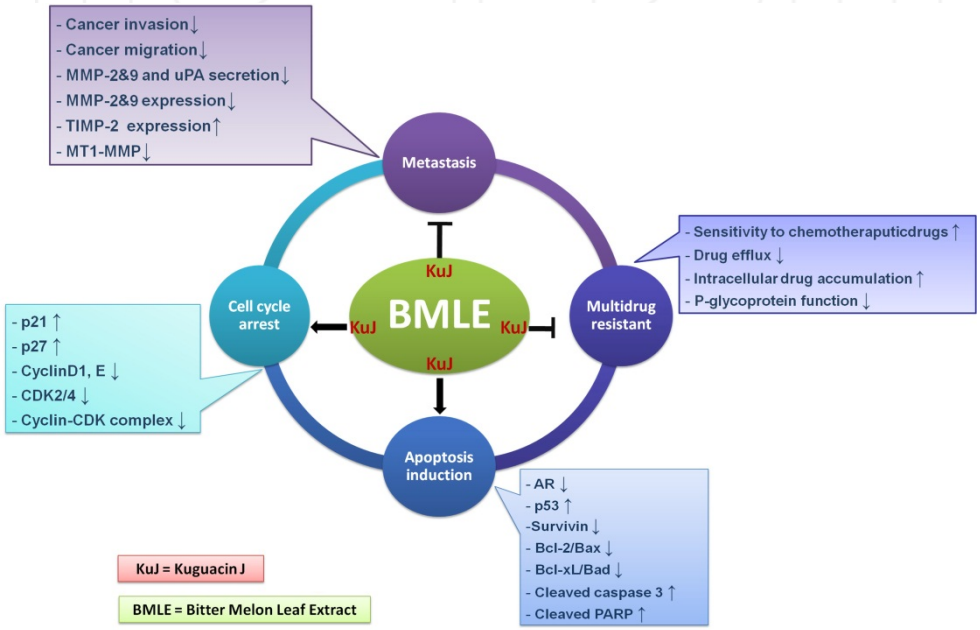

Figure 3. Summary of reported anticancer effects of bitter melon leaf extract and kuguacin J [59-61,72,73].

The critical points to be investigated in future experiments include the fact that Kuguacin J accounts for only approximately 1.6% of BMLE [61]. BMLE is known to include several triterpenoids and flavoniods. Recently various triterpenoids have shown promising effects when applied as anticancer agents [104,105]. Cucurbitacin B (cucB), a triterpenoid from Cucurbitaceae vegetables also found in bitter melon seeds, caused cell cycle arrest and apoptosis induction in human colon adenocarcinoma cancer cells [53]. Additionally, Rutin, a flavonoid present in bitter melon leaves, has been reported to display growth inhibition of leukemia and ovarian carcinoma cells, with anti-invasive effects on melanoma cells [35,56-58]. Therefore, BMLE may include other bioactive compounds apart from kuguacin J, which exert anti-tumor effects, although human studies have not yet been published. Thus, a characterization of other active components present in BMLE needs to be further elucidated.

In addition, the absorption and metabolism of the bioactive compounds after consumption remains to be investigated as to whether a test tube study can be applicable to people.

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