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Moringa oleifera and Autophagy: Evidence from *In Vitro* Studies on Chaperone-Mediated Autophagy in $HepG_2$ Cancer Cells

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ABSTRACT

Hepatocellular carcinoma (HCC) is the most prevalent primary liver cancer in Sub-Saharan African countries, including South Africa (SA). Given the limitations in current HCC therapeutics, there is an increasing need for alternative adjuvant therapeutic options. As such, several cell survival mechanisms, such as autophagy, have been identified as potential adjuvant therapeutic targets in HCC treatment. Of the three most established autophagic pathways, the upregulation of chaperone-mediated autophagy (CMA) has been extensively described in various cancer cells, including HCC cells. CMA promotes tumor growth and chemotherapeutic drug resistance, thus contributing to HCC tumorigenesis. Therefore, the modulation of CMA serves as a promising adjuvant target for current HCC therapeutic strategies. Phytochemical extracts found in the medicinal plant, *Moringa oleifera* (MO), have been shown to induce apoptosis in numerous cancer cells, including HCC. MO leaves have the greatest abundance of phytochemicals displaying anticancer potential. However, the potential interaction between the pro-apoptotic effects of MO aqueous leaf extract and the survival-promoting role of CMA in an *in vitro* model of HCC remains unclear. This review aims to summarize the latest findings on the role of CMA, and MO in the progression of HCC.

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Introduction

Globally, cancer is the second leading cause of death, accounting for approximately 8.2 million deaths in 2012 and 8.8 million deaths in 2015 (1, 2). In 2020, 19.3 million new cancer cases and 10 million cancer-related deaths were recorded worldwide (3). New cancer cases are predicted to rise to more than 23 million by the end of this decade (4). African countries currently account for an estimated 60% of all new cancer cases worldwide (1). Amongst the 36 common types of cancers worldwide, primary liver cancer was reportedly the sixth most common and the second largest contributor to cancer-related mortalities (830,000 deaths) in 2020 (3). HCC is the most common type of primary liver cancer, accounting for 75–90% of all liver cancer cases (5). Although HCC is the fifth most common type of cancer in men and the seventh most frequent form of cancer in women, it currently serves as the third leading cause of cancer-related deaths owing to the poor clinical outcomes associated with this disease (5–7). The objective of the review is to shed light on the growing burden of HCC in the continued absence of a known cure. Additionally, the review aims to explore the potential of targeting specific molecular pathways involved in tumor growth and chemotherapeutic drug resistance as a promising adjuvant therapeutic approach for treating HCC. This approach involves investigating the utilization of a medicinal plant that has demonstrated cancer cell death potential in *in vitro* models of HCC.

HCC in Sub-Saharan African Countries

The regions with the highest incidence of liver cancer include East Asia, sub-Saharan Africa (SSA), as well as north and eastern European countries (5, 8). While developed countries are also affected by HCC, this disease remains more prominent in developing countries (3, 5, 8). SSA is the most cancer-affected region, particularly by HCC (9). In 2018, African countries collectively recorded 64 779 new HCC cases, with 43 530 of these cases being male (67%) and accounting for a cumulative 63 562 deaths (98% mortality) (10).

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The regional incidence rate of HCC is amongst the highest in South Africa (SA) and its neighboring countries (9). A total of 2 710 of the 64 799 new HCC cases in Africa were from Southern Africa, resulting in 2 597 deaths (95% mortality) (10). Socio-economic factors also significantly influence the incidence of HCC, with low-income households in rural residents being at a higher risk (8). However, due to the healthcare challenges which continue to hinder the detection of early-stage HCC, current epidemiological data in SSA is thought to be an underestimation of the true scale of the prevalence and impact of HCC (9). Early-stage HCCs are usually undetectable using conventional clinical diagnostic tools (e.g., serum markers and radiography), thus making disease diagnosis difficult at this stage (11). As with many SSA countries, early-stage HCC diagnostic challenges in SA are also compounded by healthcare resource limitations (9, 12). In contrast to developed countries, most South African public hospitals have limited diagnostic resources, which include: a lack of imaging technologies used in the identification of early-stage HCC, an insufficient number of HCC healthcare specialists, and ill-equipped medical infrastructure and diagnostic laboratories (12).

HCC Risk Factors

The initiation and development of HCC is a complex process that involves numerous genetic and epigenetic events (13). These events are often hallmarks of conditions that promote the proliferation of cancer cells, while concurrently inhibiting apoptosis (13). Liver diseases and conditions such as chronic hepatitis and alcohol-induced liver damage cause liver fibrosis through epigenetic changes (5, 13–15). Epigenetic changes associated with hepatocarcinogenesis include DNA methylation and histone modifications (13, 14). Irregular DNA methylation associated with HCC development contributes to changes in signaling pathways that play a critical role in the regulation of cell cycle progression and cell death (16, 17). These include changes in the retinoblastoma 1 (RB1), p53, and wingless/int-1 (Wnt) pathways (16, 17). DNA methylation is a process that can impact gene expression and various cellular processes (18). It has been linked to DNA repair and efficacy, decreased effectiveness of treatments, and interruptions in interferon-alpha signaling – a signaling molecule crucial for the immune response against viruses and tumors (17). The histone modifications include an increase in histone deacetylation activity, changes in cellular gene expression, and the downregulation of CYP2E1, a drug-metabolizing enzyme that is primarily expressed in hepatocytes (17, 19). Findings by Herceg and Paliwal have also revealed that RNA interferences may also be associated with HCC development (17). These epigenetic changes eventually develop into cirrhosis, which is one of the most common precursors for HCC (13, 14, 17). Cirrhosis results from late-stage liver disease and its associated complications (14). Hepatitis B virus (HBV) and hepatitis C virus (HCV) are also major contributors to the high incidence of HCC, with HBV being the primary underlying cause for an estimated 60% of all HCC cases in developing countries (5, 20). Other significant contributors to primary liver cancer include obesity, type II diabetes mellitus, alcohol-related liver disease, and nonalcoholic fatty liver disease (5, 8, 15, 20). The human immunodeficiency virus, of which SA has the highest number of cases worldwide, is also considered a prominent risk factor for the development of HCC (8, 21). Additional risk factors include liver metastasis, ethnicity, age, diet, gender, and geographic location (9, 13, 15, 22). The varying HCC epidemiological disparities across different regions of the world are further observed between populations within the same region (13, 15).

The Molecular Pathways Implicated in HCC Progression

Selective cell signaling pathways, crucial in maintaining cellular homeostasis, have been identified as promising adjuvant therapeutic targets in the treatment of HCC (13). However, much remains unclear about the complex mechanistic role that these pathways may play in hepatocarcinogenesis (13). Most prominent is the role of the autophagy intracellular pathways, which have been implicated in various human diseases, including age-associated neurodegenerative diseases, cardiovascular diseases, liver disorders, as well as in the pathogenesis of HCC (23–27). Under physiological conditions, the autophagy pathway, with the term "auto" meaning self and "phagy" meaning to eat, is a cell survival mechanism that results in the delivery of cytoplasmic components, including damaged organelles, and aggregated or superfluous proteins to the lysosome for subsequent degradation (28–30). Autophagy plays a crucial role in cellular processes such as development, differentiation, cellular stress response, and is involved in the overall maintenance of cellular homeostasis, in both the absence and presence of various stress conditions (31, 32). Under physiological conditions, cytoplasmic components, including mitochondria and proteins, become damaged and aggregate over time, thus necessitating their removal

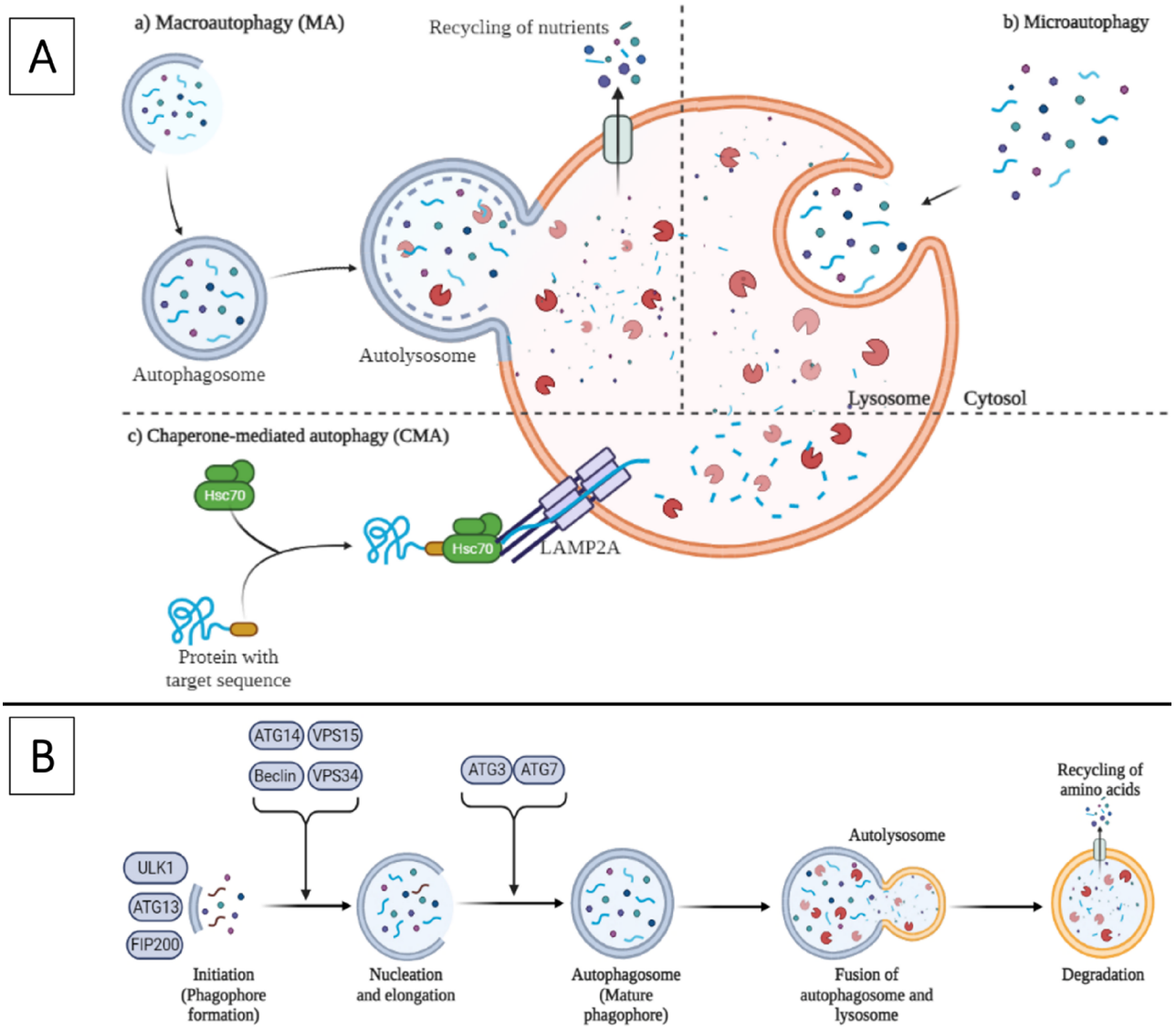

Figure 1. (A) A schematic representation of the three major autophagy pathways: macroautophagy (MA), microautophagy and chaperone-mediated autophagy (CMA). (B) A schematic representation of the stepwise MA pathway. ATG, autophagy-related protein; FIP200, FAK family-interacting protein of 200 kDa; ULK, UNC-51 like autophagy activating kinase 1 (Adapted from Griffey and Yamamoto (49), Xu et al. (44)).

(33). The continuous removal of defective and toxic cytoplasmic components through the different types of autophagy pathways prevents the accumulation thereof, thus averting the development of various human diseases and ensuring the continual renewal and reuse of cytoplasmic material by the cell (25, 34).

Autophagy Pathways

Autophagy is primarily sub-classified into three major pathways: namely, macroautophagy (MA), microautophagy and chaperone-mediated autophagy (CMA) (Figure 1A) (28, 35, 36). Of the three autophagic pathways, MA and CMA are the most recognized and extensively described pathways, as both pathways play key roles in maintaining cellular homeostasis, and their dysfunction has been characterized in many diseases, including such as neurodegeneration, cancer, and inflammatory disorders (37–39).

The Macroautophagy Pathway

Macroautophagy is a complex process characterized by the de *novo* synthesis of a unique double-membrane structure, called the autophagosome (40). This cellular pathway involves the coordinated action of more than 40 autophagy related (ATG) proteins (41, 42). The initiation of MA involves the assembly of the uncoordinated-51 (UNC-51)-like autophagy activating

kinase (ULK) complex, which includes ULK1, ATG13, the 200-kDa FAK-family interacting protein (FIP200), and ATG101 at the location of the isolation membrane, where autophagosome formation is initiated together with the recruitment of other ATGs (Figure 1B) (43, 44). The activity of MA is regulated by cellular stress conditions such as nutrient deprivation, hypoxia, or cellular stress, which activate upstream signaling pathways that lead to the inhibition of the mammalian target of rapamycin complex 1 (mTORC1) (37, 38, 45). In general, mTORC1 inhibits MA by promoting protein synthesis and inhibiting protein degradation, which reduces the need for cells to carry out MA when nutrients are abundant in the cytosol. When mTORC1 is active, it phosphorylates and inhibits the ULK1 complex, which is a key complex of proteins involved in the initiation of MA (46, 47). Conversely, when mTORC1 is inhibited under stress conditions, this allows for the activation of ULK1 by mTORC1 through the phosphorylation of a specific site on ULK1, known as Ser757 (47). This phosphorylation event leads to the dissociation of ULK1 from its negative regulator AMP-activated protein kinase (AMPK), and subsequently, the initiation of MA (48).

The step-by-step process of MA can be summarized as follows: (1) the initiation and formation of an isolation membrane, called the phagophore, (2) the nucleation and elongation of the phagophore to form the autophagosome, which elongates, surrounds, and engulfs selective cytoplasmic components, (3) the maturation and fusion of the autophagosome with the lysosomal membrane to form the autolysosome, and (4) the degradation of autolysosomes, which leads to the release and recycling of the engulfed cytoplasmic materials (Figure 1B) (37, 49, 50). The products of MA degradation, such as amino acids, are recycled back to the cytosol where they can be used by the cell for energy or to synthesize new proteins (51). When amino acids become abundant in the cytosol, they can exert negative feedback on MA.

In brief, once activated, the ULK1 complex induces the recruitment of the class III phosphatidylinositol-4,5-bisphosphate 3-kinase complex (PI3KC3), which includes ATG14, Beclin 1, p63, and AMBRA1 (autophagy and beclin 1 regulator 1) to the phagophore membrane (26). The phagophore is a double-membrane structure made up of various plasma membrane sources from the cytoplasm such as the ER, lysosome, and endosomes (37). The two sides of the phagophore arc fuze around the cytoplasmic cargo material, forming the autolysosome (52). The recruitment of specialized ATGs to the phagophore membrane promotes phagophore membrane nucleation and the recruitment of other ATGs to the membrane of the pre-autophagosome structure, to promote elongation and expansion, and eventually the completion of autophagosome structure (53–55).

During the elongation and expansion steps, ATG7 and ATG10 facilitate the formation of the ATG5-ATG12 complex, which conjugates with ATG16L1 to form the ATG5-ATG12-ATG16L1 multimeric complex (56). Together with ATG3, ATG4 and ATG7, the ATG5-ATG12-ATG16L1 complex mediates the conjugation of phosphatidylethanolamine (PE) to the microtubule-associated protein 1 light chain 3 (LC3)-I to form LC3-II (28, 57). This conjugation reaction leads to the cytoplasmic translocation of LC3 to the membrane of the pre-autophagosomes, which together with the GABA type A receptor-associated protein (GABARAP) families, is necessary for the formation and maturation of autophagosomes (28, 52, 57).

Once matured, the autophagosomes move to the perinuclear region, where most autophagosome-lysosome fusion occurs. This movement involves a series of coordinated steps that are mediated by proteins and molecular motors along microtubule tracks toward the lysosomes (51). Once the autophagosomal and lysosomal membranes have fused, the contents of the autophagosome are exposed to the hydrolytic enzymes within the lysosome, which mediate the degradation of the sequestered cytoplasmic material. The resulting breakdown products are recycled back to the cell (58).

The Microautophagy Pathway

Unlike MA, which involves the formation of double-membrane autophagosomes to sequester cytoplasmic components (52), microautophagy is a non-selective lysosomal degradative process that involves the direct invagination of the lysosomal membrane to engulf cytosolic components (59–61). Uptake occurs directly at the limiting membrane of the lysosome and can include intact organelles (60, 62). This cellular process has been observed in yeast and mammalian cells (39). Microautophagy is induced by a variety of stimuli, including nutrient deprivation, oxidative stress, and hypoxia (39).

Although the different sequestration mechanisms of the microautophagy pathways require further elucidation, two types of cargo uptake mechanisms have been widely accepted (Figure 2): (i) fission-type microautophagy and (ii) fusion-type microautophagy (63, 64). Fission-type microautophagy (Figure 2(i)), involves the formation of membrane-bound vesicles or tubules that bud off from the lysosomal membrane and sequester the cargo to be degraded (64). The

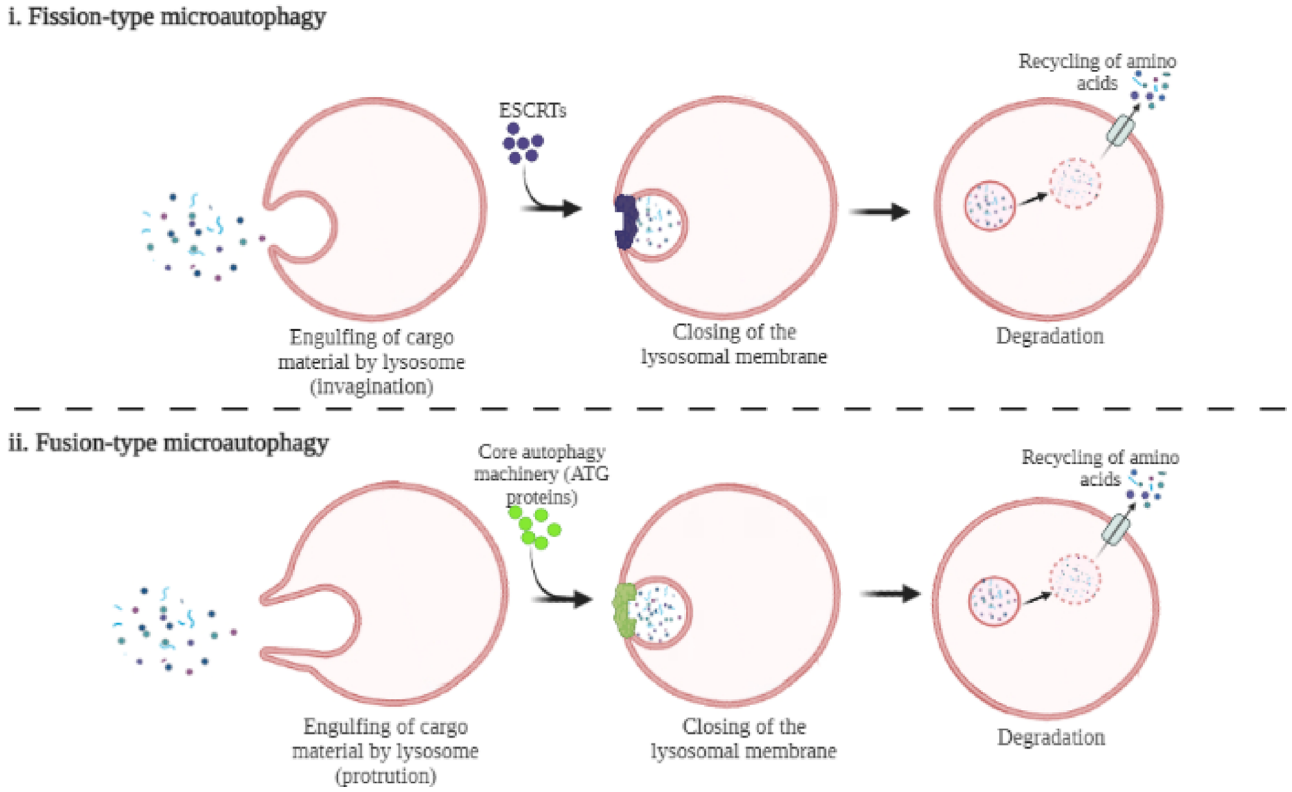

Figure 2. A schematic representation of the stepwise microautophagy pathway. ATG, autophagy-related; ESCRT, endosomal sorting complex required for transport (Adapted from Schuck (63)).

vesicles or tubules then undergo fission, releasing their contents into the lysosomal lumen for degradation (64, 65). This type of microautophagy is mediated by endosomal sorting complexes required for transport (ESCRTs) (63, 64). ESCRTs are required for endosomal microautophagy (eMI) which is a selective branch of microautophagy that involves late endosomes (LE)/multivesicular bodies (MVB) (65). Endocytic vesicles containing cargo proteins develop into MVBs (66). The ESCRT machinery members are required for the invagination and closing of the MVBs following the engulfment of the cargo proteins (65, 67). Fusion-type microautophagy (Figure 2(ii)), relies on the direct fusion of lysosomes with the substrate to be degraded. Increasing evidence suggests core ATGs may be involved in fusion-type microautophagy, which is thought to play a role in a variety of cellular processes, including protein quality control, lipid metabolism, and signaling pathways (64, 68). The accumulation of experimental data in the past decade has led to the mechanisms that regulate microautophagy becoming more appreciated (39, 64). However, further research is warranted to further shed insights into its physiological roles in cellular homeostasis and disease.

The Chaperone-Mediated Autophagy (CMA) Pathway

Unlike microautophagy and MA, the CMA pathway does not use vacuolar structures to sequester cargo, but instead involves the use of chaperone proteins that selectively recognize, bind, and transport specific cytosolic proteins to the lysosome for subsequent degradation (69). Similar to MA, CMA activity is upregulated in response to prolonged starvation conditions, thus ensuring the continuous degradation of nonessential proteins, and providing nutrient reserves for the cell while also preserving key proteins (23). While the role of MA in cancer has been extensively described, the role of CMA has more recently gained increasing research interest. Although the role of CMA in numerous cancers has been well-established (70–72), there is a noticeable scarcity of of literature focusing on its specific modulation and potential therapeutic exploitation in the context of HCC treatment strategies. Pioneering studies by Zhang and Cuervo were the first to report on the relationship between CMA, aging, and liver function (31). Using CMA-defective aged mice, the study found that restoring CMA function in the liver of aging mice led to a reduction in the accumulation of damaged proteins in liver cells,

an improvement in liver function, and an increase in mice lifespan (31). Conversely, the blockage of liver CMA was shown to lead to hepatosteatosis and altered glucose metabolism in a mouse model with defective hepatic CMA (73). These studies were amongst the first to demonstrate the important implications for the development of CMA-based therapies related to various liver diseases and maintaining hepatic function. More recently, Schneider and colleagues demonstrated the prominent role of CMA in liver cancer (74). The authors used genetically modified mouse models, and human liver tissues and cells to investigate the role of CMA and MA in the development of HCC in cirrhotic liver cells. They found that increased CMA activity compensated for impaired MA in cirrhotic liver cells (74), suggesting that CMA may compensate for impaired MA in the liver by degrading specific proteins that are involved in tumor suppression. The study also found that the loss of a key CMA receptor protein in mice with liver cirrhosis resulted in a reduction in HCC development, suggesting that CMA contributes to HCC promotion in cirrhotic livers (74). Moreover, CMA-targeted therapy was found to reduce HCC development in a mouse model of liver cancer. The upregulation of CMA has also been extensively described in other cancer cells, including skin, lung, and other types of liver cancer cells (24, 75). In particular, the increased expression of the CMA-specific receptor, lysosome-associated membrane protein type 2 A (LAMP2A), has been shown to augment HCC tumor growth and enhance tumor recurrence (76). This suggests that elevated protein expression levels of key CMA markers may be associated with a worse prognosis for HCC patients (74, 76). Taken together, these studies suggest that the downregulation of CMA activity may be exploited as an adjuvant target in HCC therapeutic strategies.

Mechanistic Overview of CMA

The CMA pathway uses a chaperone-recognition system that allows for specific proteins to be 'chaperoned' by a cytosolic chaperone complex to the lysosomal membrane for subsequent degradation (Figure 3) (77). CMA specifically targets and degrades cytosolic proteins containing the Lysine (K) - Phenylalanine (F) -Glutamic Acid (E) - Arginine (R) - Glutamine (Q) (KFERQ) pentapeptide sequence (78). In immunochemistry studies, the KFERQ pentapeptide sequence found within selective cytosolic proteins is always exposed, thus making it easily accessible for recognition and binding (77). Such proteins constitute approximately 30% of all cytosolic proteins (79). The KFERQ-harboring protein is recognized and bound by the heat shock cognate 71 kDa protein (HSC70), which forms part of a chaperone complex (77–79). The chaperone complex also consists of heat shock protein 40, heat shock protein 90 (HSP90), HSC70-HSP90 organizing protein and B-cell lymphoma 2 -associated athanogene (77, 79). Once bound, the cargo protein (i.e., the targeted KFERQ-harboring protein) is transported by the chaperone complex and delivered to the lysosomal membrane (77, 78). Subsequently, the chaperone complex binds to the cytosolic tail of LAMP2A, thereby triggering the unfolding of the cargo protein and aiding in the protein's translocation across the lysosomal membrane (77–79). During this translocation process, an HSC70-associated chaperone protein, HSP90, helps to keep the LAMP2A-protein interaction stable (78). HSC70 then facilitates the disassembly of the chaperone complex once the translocation process is completed, thus allowing LAMP2A to return to its natural monomeric state (78). The translocation of the cargo protein into the lysosomal lumen is further aided by the lysosomal variant of HSC70 (lys-HSC70), which is necessary for the complete translocation of the cargo protein (78). The degradation and organization (assembly and disassembly) of LAMP2A at the lysosomal membrane determines how much viable LAMP2A is available for additional cargo protein binding (Figure 3) (78). The binding of LAMP2A to the substrate protein is thus defined as the rate-limiting step for CMA activity, with the expression levels of LAMP2A at the lysosomal membrane being directly correlated with the level of CMA activity (80, 81).

The Regulation of LAMP2A Dynamics at the Lysosomal Membrane

The binding of the chaperone complex with the cytosolic tail of LAMP2A triggers the multimerization of LAMP2A, thereby forming a multimeric complex that mediates the translocation of the substrate protein into the lysosome (82, 83). The stability of the LAMP2A multimeric complex is regulated by two proteins in a guanosine triphosphate (GTP)-dependent manner: namely, glial fibrillary acidic protein (GFAP) and elongation factor 1α (EF1α) (84). The multimerization of LAMP2A triggers a series of events at the lysosomal membrane: (1) GFAP transiently binds with LAMP2A, thereby stabilizing it in its multimeric conformation, and (2) the second regulatory protein, EF1α, binds to a phosphorylated variant of GFAP (pGFAP) which is also present at the lysosomal membrane (82, 83, 85). Following the successful

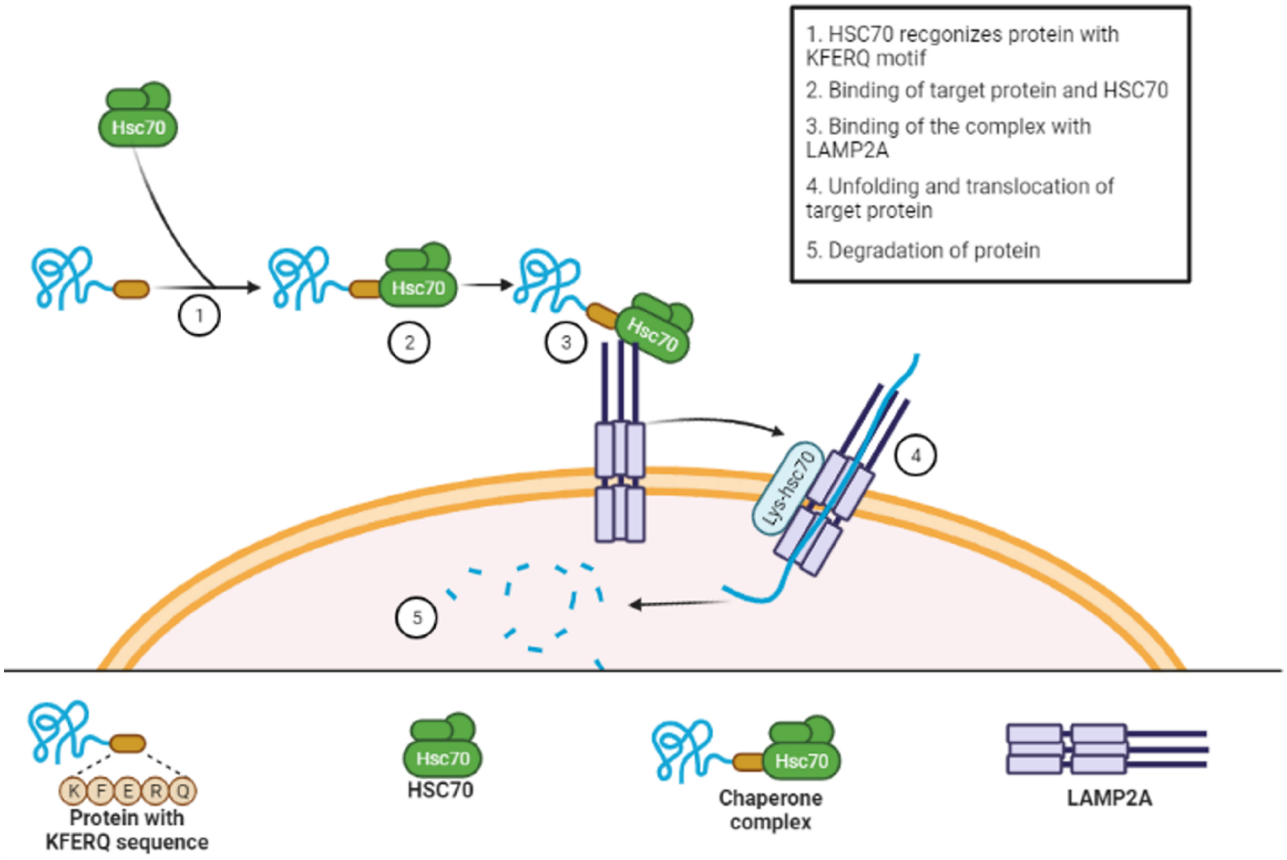

Figure 3. A schematic representation of the stepwise chaperone-mediated autophagy pathway. HSC70, heat shock cognate 71 kDa protein; LAMP2A; lysosome-associated membrane protein type 2 A; lys-HSC70, lysosomal variant of HSC70.

translocation of the substrate protein and the presence of GTP, the lysosomal membrane releases EF1α from pGFAP, thus exposing the pGFAP binding site (86, 87). GFAP has a stronger affinity for pGFAP than it does to LAMP2A; the release of EF1α, therefore, causes GFAP to unbind with the multimeric LAMP2A and bind to pGFAP instead (85–87). The release of GFAP from the multimeric LAMP2A-chaperone complex results in the destabilization of LAMP2A by cytosolic HSC70, causing it to revert to its monomeric conformation (83, 86, 87). This causes a decrease in CMA activity which in turn allows the cycle to be repeated (82, 87).

The role of Lysosomal mTORC2/PHLPP1/Akt Signaling Axis in CMA

One of the mechanisms through which cells regulate metabolism and evaluate the cell's nutritional status is primarily through the activity of mTOR, which senses and integrates different nutritional inputs including cellular stress, amino acids, and energy levels (82, 88). There are two mTOR complexes namely: mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2) (82, 88). While the activation of mTORC1 results in the inhibition of MA activity, mTORC2 and its effector protein kinase B (Akt), specifically Akt1, is almost exclusively detected in CMA-competent lysosomes where mTORC2/Akt1 negatively regulates the assembly of LAMP2A into the CMA translocation complex (Figure 4) (82, 86). Under basal CMA activity, mTORC2 phosphorylates Akt1 which in turn phosphorylates GFAP at the lysosomal membrane (87, 89). According to findings by Catarino and colleagues, Akt activity, which is activated by mTORC2, in turn, controls the level of pGFAP (87). The continuous activity of mTORC2/Akt1 represses CMA activation by negatively regulating the assembly of LAMP2A into the translocation complex and thereby contributing to the relatively low basal levels of CMA activity (82, 86). However, under cellular stress conditions such as prolonged starvation, when the upregulation of CMA activity is required, the activity of mTORC2 is opposed by the recruitment of pleckstrin homology domain and leucine-rich repeat protein phosphatase 1 (PHLPP1) to the lysosomal membrane (82, 83, 87,

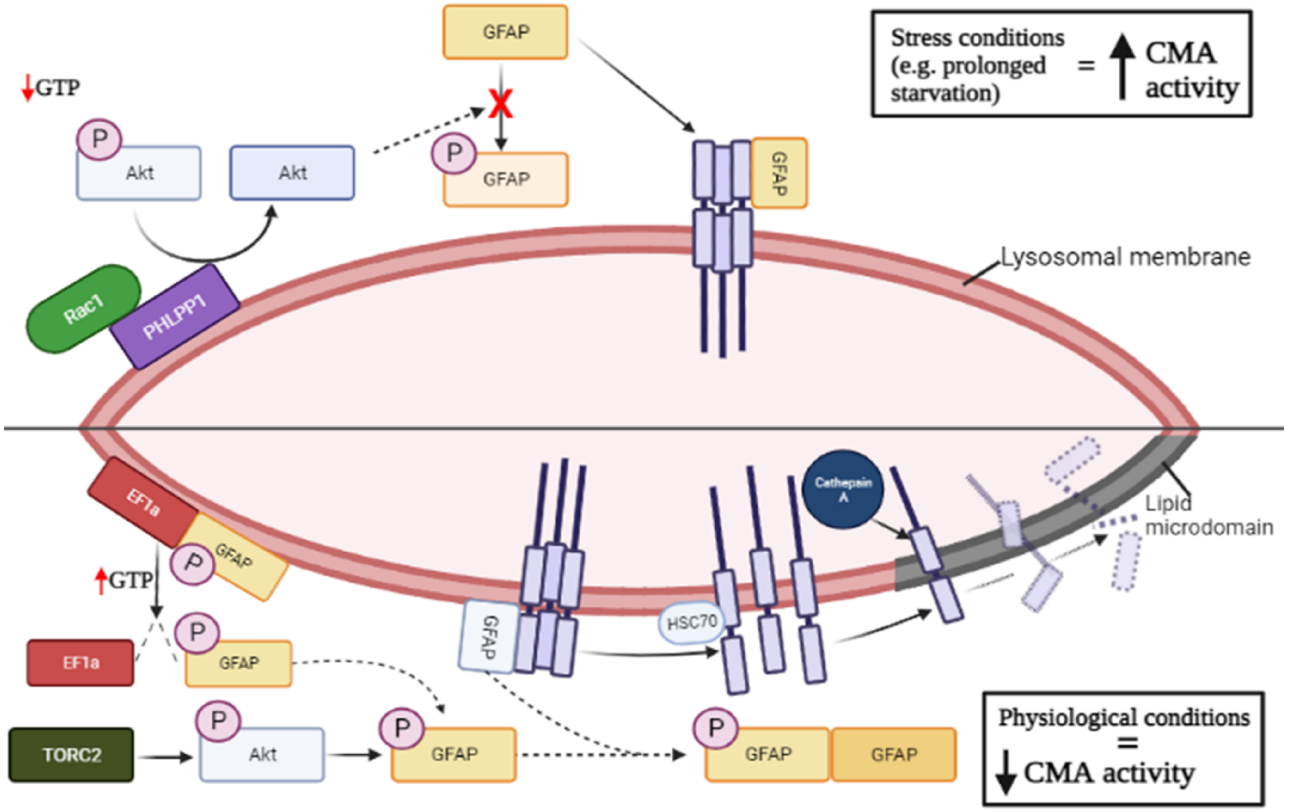

Figure 4. The regulation of CMA activity by the mTORC2/PHLPP1/Akt signaling axis. Akt, Protein kinase B; EF1a, Elongation factor 1α; GFAP, Glial fibrillary acidic protein; GTP, Guanosine triphosphate; HSC70, Heat shock cognate 71 kDa protein; PHLPP1, Pleckstrin homology domain and leucine-rich repeat protein phosphatase 1; Rac1, Rat sarcoma virus-related C3 botulinum toxin substrate 1; TORC2, Target of rapamycin complex 2 (in mammals: mammalian target of rapamycin complex 2, mTORC2) (Adapted from Fu and Hall (88)).

89). At the lysosomal membrane, PHLPP1 is stabilized by the GTPase rat sarcoma virus-related C3 botulinum toxin substrate 1 (Rac1) (82). PHLPP1 is responsible for dephosphorylating Akt1; the deactivation of Akt1 activity increases the pool of non-phosphorylated GFAP, thus favoring the formation of the CMA translocation complex (82, 83, 87, 89). CMA activity is thus regulated by the mTORC2/PHLPP/Akt signaling axis through its activity on the CMA translocation complex at the lysosomal membrane (Figure 4) (82, 83).

Findings from a study by Bandyopadhyay and colleagues revealed that mechanistically, the inhibition of CMA by GTP occurs through its effect on EF1α (84). GTP induces the release of EF1α from its complex with pGFAP at the lysosomal membrane, subsequently causing GFAP to be released from LAMP2A (84, 87). The role of GTP in CMA regulation has been further highlighted in a study by Catarino and colleagues, where GTP was also shown to inhibit both the recruitment of PHLPP1 to the lysosomal membrane and the stabilization of PHLPP1 by Rac1 (87).

The Dual Role of CMA in Cancer

The role of CMA in cancer is complex and context-dependent. While CMA has been shown to act as a tumor suppressor in some contexts, it can also promote cancer cell survival and growth under stress conditions, such as nutrient deprivation, hypoxia, and chemotherapy (42, 90). Further research is needed to fully understand the role of CMA in cancer and to identify potential therapeutic targets for cancer treatment.

The Pro-Death Role of CMA in Cancer

The primary function of CMA involves the maintenance of cellular quality control through its role in the regulation of cellular metabolism (91, 92). CMA's quality control function contributes to the prevention of malignant cell transformation in untransformed cells (91). However, it is also associated with promoting cancer cell proliferation (24, 91). CMA has also been shown to play a continuous role in the cell cycle process through activities such as the regulation of

DNA repair (70). Basal CMA activity levels are elevated in organs that have higher gluconeogenesis rates such as the liver (89). According to Kaushik and Cuervo, despite the compensatory role of other autophagy pathways such as macroautophagy, aging and cellular stressors such as oxidative stress still result in proteotoxicity, due to the aggregation of damaged proteins in CMA-compromised tissues (89). In an earlier study, Tang and colleagues also demonstrated that the selective activation of CMA promotes cancer cell death by targeting and degrading selective oncogenic proteins (93). Consistent with these findings, in 2015, Galan-Acosta and colleagues first reported the role of hexokinase 2 (HK2) as a CMA amiable target (94). HK2 acts as both a glycolytic enzyme and an oncogenic kinase and is overexpressed in cells with a relatively high glucose metabolism rate, which is indicative of most cancer cell types (94–96). Under physiological conditions, HK2 plays a key role in the first committed step of glycolysis (glucose metabolism) by catalyzing the phosphorylation of glucose (94, 96). Due to its oncogenic kinase function, HK2 promotes glycolytic activity in cancer cells and thereby contributes to the initiation and survival of several cancerous tumors, including breast and lung cancer (94, 96, 97). The overexpression of this protein has been associated with the ability of cancer cells to maintain growth factor-independent glucose metabolism, and to avoid cell death in the absence of growth factors (94). In addition, the expression of HK2 in HCC tumors has been associated with worse patient outcomes (98, 99). The ability of CMA to target and degrade HK2, and the role of HK2 in cancerous cells, suggests that CMA activity can potentially be modulated to promote cancer cell death (94).

The Pro-Survival Role of CMA in Cancer

The role of CMA in cancer is complex, with both tumor-suppressive and pro-survival effects. Under normal physiological conditions, CMA has been shown to suppress cancer by removing and degrading specific proto-oncogenic proteins (92). Impairment of CMA can lead to the accumulation of these tumor-inducing proteins, creating favorable conditions for cancer development (92, 100, 101).

Although CMA has a well-established physiological role, an increasing number of studies also indicate that CMA activity is elevated and adopts a pro-survival role in most cancer cells studied to date (24, 102). A decrease in cancerous cell growth has been reported following the concentration-dependent inhibition of CMA activity in response to retinoic acid (RA) treatment, as evidenced in leukemia, breast, and liver cancer cells (103–105). Studies have demonstrated elevated CMA activity in human primary tumor tissues compared to normal tissues, along with an increase in the expression of LAMP2A, a key CMA protein, in tumor tissues (24). The study further revealed that inhibition of CMA reduced cancer cell proliferation, as observed in human lung cells where LAMP2A was knocked down (24). Studies have shown that CMA can promote cancer cell survival and drug resistance by protecting cancer cells from the cytotoxic effects of chemotherapy (106, 107). CMA also contributes to the maintenance of the Warburg effect, a metabolic characteristic of cancer cells (24, 70). Suzuki and colleagues also explored the role of CMA in cancer survival, revealing that CMA inhibits the degradation of Myeloid leukemia 1 (a key cancer pro-survival protein), and stabilizes this protein in non-small-cell lung cancer cells (108). In breast cancer cells, CMA has been shown to protect cells from the cytotoxic effects of the chemotherapy drug doxorubicin by degrading the pro-apoptotic protein BCL2-interacting mediator of cell death (Bim), which is normally upregulated by doxorubicin to induce apoptosis (109). Similarly, in lung cancer cells and esophageal squamous cell carcinoma, CMA has been shown to affect tumor cell proliferation and protect cells from the cytotoxic effects of the chemotherapy drug cisplatin by degrading the tumor suppressor protein p53, which is normally upregulated by cisplatin to induce cell death (110, 111). Overall, the findings suggest that targeting CMA could be a potential strategy to overcome cancer treatment resistance. However, further research is needed to fully understand the role of CMA in treatment resistance and to identify potential modulators of CMA for therapeutic use.

Modulators of CMA Activity

Under physiological conditions, CMA activity remains at a relatively low level in most cell types, including hepatocytes (82, 86). Under basal CMA activity, LAMP2A is mobilized to specific lipid microdomains on the lysosomal membrane where it is cleaved by Cathepsin A and degraded by lysosomal enzymes upon binding (Figure 4) (87, 112).

However, when CMA activity is upregulated, the elevated levels of LAMP2A multimerization during substrate translocation ensures that most proteins are excluded from the microdomains (87, 112). Additional cellular stressors such as oxidative stress, lipotoxicity, and proteotoxicity have also been shown to elevate the level of CMA activity (82, 86, 113). External factors

such as age may also alter the cell-dependent levels of basal CMA activity, as CMA activity has been shown to decrease with increasing age in most cells (112, 113). Changes in the dynamic degradation of LAMP2A at the lipid microdomains have been shown to be the main contributor to the age-related decline in CMA activity (112). Moreover, other mechanisms such as the mobilization of LAMP2A from the lysosomal lumen to the lysosomal membrane, the degradation of LAMP2A, and the synthesis of LAMP2A proteins also modulate the cellular level of CMA activity (112).

Chemical Modulators of CMA Activity

Due to its dual role in cancer progression, the ability to selectively target CMA on a cellular level could potentially be a promising adjuvant therapeutic strategy (114). To this end, the selective modulation of CMA activity using various chemical compounds has become a topic of increasing research interest (114). Chemical modulators that have been shown to selectively modulate CMA activity in various cell types are summarized in (Table 1).

Chemical modulation of CMA activity by retinoic acids

Retinoic acids have shown significant promise in the selective modulation of CMA activity when compared to other chemical modulators in numerous cancerous cells assessed to date (103, 114). The molecular mechanism through which RA inhibits cancerous cell growth is thought to involve the retinoic acid receptor alpha (RARα) mediated signal transduction (115). The RARα pathway is the only ubiquitous RAR pathway in mammalian cells that has been successfully exploited for its selective inhibition of the CMA pathway (103, 114). To this end, the RARα activator, all-trans-retinoic acid (ATRA), has been therapeutically exploited to target the RARα pathway (103). In a study by Lee and colleagues, ATRA was shown to activate mTORC2 and its effector protein kinase, Akt1, which has been shown to inhibit CMA activity (82, 116). ATRA thus serves as a promising compound to investigate the cellular effects of CMA inhibition.

Table 1. Cell-specific chemical modulators of CMA activity (Adapted from Parzych and Klionsky (78)).

Compounds	Target	Effect on CMA
Cycloheximide	Protein synthesis inhibitor	Inhibition
Anisomycin	Protein synthesis inhibitor	Inhibition
SB230580	P38 MAPK inhibitor	Inhibition
Geldanamycin	HSP90 inhibitor	Activation
17-AAG/DCA	HSP90 inhibitor + PDK1 inhibitor	Activation
6-aminonicotinamide	G6PDH inhibitor	Activation
Synthetic ATRA derivatives	RAR-alpha inhibitor	Activation
Torin	TORC2 inhibitor	Activation
TAK165/AC220	MA inhibitor + FLT3 Inhibitor	Activation
Spautin/AC220	MA inhibitor + FLT3 inhibitor	Activation

The Effects of ATRA on Cancerous Cells

Retinoic acid derivatives have been shown to influence cellular differentiation, cellular growth, and apoptosis (117). In cancerous cells, ATRA and its derivatives (retinoids) have shown promising anti-cancer effects and display anti-proliferative, antioxidant, and cytotoxic traits that influence cell differentiation and induce apoptotic effects through the nuclear retinoic acid receptors, including RARα apoptosis (117). Studies suggest that a lack of oxygen in the tumor microenvironment may be associated with a more aggressive tumor, and increased ability of the cancerous cells to invade nearby tissues (118–121). A study by Yasamin and colleagues investigated the effects of ATRA on breast cancer using noninvasive ductal carcinoma cells (DCIS) *in situ* under hypoxic conditions (118). This study revealed that ATRA eliminated the effects of hypoxia, prevented epithelial-to-mesenchymal cell transformation, and prevented malignancy of the DCIS cells which is usually associated with a hypoxic microenvironment (118). ATRA's efficacy against cancerous cells is not limited to breast cancer. According to findings by Ni and colleagues, ATRA also induces differentiation of acute promyelocytic leukemia (APL) tumor cells, thereby significantly increasing the remission rate of ALP-affected patients (122). Interestingly, clinical examination following the administration of ATRA revealed the potential ability of this compound to selectively reverse an otherwise fatal APL case into one that is highly treatable (122). ATRA has also shown the therapeutic potential to improve the efficacy of other available leukemia therapeutics (123). This was evidenced by the improved survival of acute myeloid leukemia patients over the age of 60 who were treated with a combination of low-dose cytosine arabinoside (LDAC) and ATRA, as compared to LDAC treatment alone (123). Schenk and colleagues further reported that children with high-risk neuroblastoma treated with 13-*cis*-retinoic acid (an ATRA derivative) had an improved survival rate following high-dose chemotherapy, and stem cell transplantation (123). Other forms of cancer where ATRA activity has shown efficacy include kidney, lung, cervical, and liver cancer (22, 117).

Despite its therapeutic potential, there are several challenges with the clinical application of ATRA. One such challenge is due to ATRA's ability to promote angiogenesis within the tumor by upregulating the transcription of the vascular endothelial growth factor gene (22). Moreover, ATRA is a highly toxic compound that may become ineffective due to the high probability of tumor cells developing resistance to it (22). Some isomers and derivatives of ATRA have been shown to be more effective when administered in combination with chemotherapeutic drugs compared, to combination treatment using other isoforms of ATRA, in selective types of cancer (122).

The Dual Effects of ATRA in HCC

Clinical and *in vitro* studies have shown that ATRA affects HCC progression (22, 117, 124, 125). These studies have shown that ATRA influences the transcriptional response, and cell differentiation process of the $HepG_2$ cells (126). In a separate study, it was revealed that ATRA may promote the survival of various HCC cells, including $HepG_2$ cancer cells (125). In this study, Wang and colleagues assessed the effects of ATRA treatment on $HepG_2$, Hep_3B, and Huh7 liver cancer cells cultured without serum, and their findings revealed that ATRA treatment enabled these cells to evade starvation-induced apoptosis (125). Additionally, ATRA treatment was also found to promote the proliferation and invasion of HCC cells into the interstitial connective tissue under conditions of serum starvation (125). Furthermore, ATRA treatment upregulated the cellular extracellular matrix (ECM) genes, ultimately resulting in HCC ECM remodeling, and thus promoting the survival of the HCC cells (125). In contrast to the above findings, Wang and colleagues also reported on the anti-cancer properties of ATRA treatment in HCC, highlighting a potential dual role of ATRA exposure in HCC (125). The above findings revealed that ATRA may improve the prognosis of HCC patients and potentially influence the survival period of patients with advanced-stage HCC (125).

In agreement with the above hypothesis, findings from an increasing number of studies agree that ATRA may also exhibit HCC anti-cancer properties (22, 117, 127). In a clinical study by Zhu and colleagues, it was shown that combination chemotherapeutic treatment including ATRA inhibited the development of secondary cancers in patients with early-stage HCC (117). Consistent with these findings, *in vitro* studies have also shown the anti-cancer effects of ATRA, which include ATRA-induced inhibition of HCC cell growth (22, 117). A study by Liu and colleagues revealed that ATRA treatment inhibits the proliferation of $HepG_2$ cancer cells by increasing the expression of tumor suppressor protein, p53 (22). In line with these findings, Wei and colleagues also highlighted the anti-cancer effects of ATRA on $HepG_2$ cancer cells by demonstrating that ATRA had a time- and dose-dependent apoptotic effect on these cells (128). An earlier study by Yoon and colleagues revealed that ATRA was also able to hinder the expression of Vimentin (a growth-related gene) mRNA in Hep_3B HCC cells (127). Vimentin is associated with the progression of carcinomas (i.e., increased motility and invasive ability of the cancer cells), evasiveness of tumors against treatment, and worse patient prognosis (127). The overexpression of this gene has been associated with the metastasis of various tumor types including HCC tumors (127, 129, 130). Yoon and colleagues reported that ATRA was able to reduce the motility and invasion of the Hep_3B cells by inhibiting the expression of vimentin (127).

Aside from its various effects on HCC, ATRA treatment has also been shown to have a variety of physiological functions within the liver (126). These functions include hepatic protein metabolism, cell differentiation, and the production of albumin and urea (126).

The Role of CMA in HCC

According to Li and colleagues, CMA promotes HBV and HCV replication, thus accelerating its carcinogenic effects on the liver (131). HCV has been reported to interact with the HCV nonstructural protein 5 A (NS5A), which plays an important role in the HCV RNA replication and the regulation of the virus' assembly (132, 133). This interaction subsequently induces the selective targeting and degradation of transcription factor hepatocyte nuclear factor 1α (HNF-1α) protein *via* the CMA pathway (132). This degradation has been associated with HCV-induced pathogenesis (132). HCV-CMA has also been linked with a decrease in beclin 1, a mammalian protein that can inhibit tumorigenesis, and has also been associated with a poorer prognosis (83, 134–136). Ding and colleagues assessed CMA activity in different human HCC cell lines, including the $HepG_2$, Hep_3B, Huh7, MHCC97L, MHCC97H, and HCCLM3, and further compared their findings to the level of CMA activity in the normal human hepatic cell line L-02 (76). This study revealed that a high LAMP2A expression was not only associated with larger HCC tumor size and improved overall tumor survival, but it was also associated with a higher rate of tumor recurrence

(3-, 5- and 7-year cumulative recurrence rates) in HCC patients (76). Additionally, the inhibition of LAMP2A in HCC tumors decreased tumor cell viability and proliferation under stressful conditions (76). These findings suggest that the long-term survival of HCC tumor cells may depend, at least in part, on the presence of a high level of CMA activity. CMA has also been shown to contribute to the development of chemotherapeutic resistance in HCC cells (137). This finding was evidenced by the reduction of cyclin D1 expression, a protein previously shown to interact with tumor-suppressor proteins as a response to increased CMA activity (137), thus reducing HCC cell apoptosis.

Studies have shown that upregulated CMA in cancerous cells aids in the removal of excessive free radicals, reducing ROS production (138). The free radicals produced by oxidative stress can lead to cellular damage (139). In cancerous cells, including HCC cells, the excess level of oxidative stress contributes to the upregulation of CMA (139, 140). There upregulated activity of CMA helps the cancerous cells remove components damaged by the excessive ROS levels, allowing the cells to avoid oxidative stress-linked apoptosis (138, 139). These findings provide compelling evidence for the pro-cancer role in the presence of enhanced CMA activity, suggesting that CMA may be a potential target for novel HCC therapeutics or as part of a combination therapy approach (24, 76).

Treatment Options for HCC

The treatment of HCC depends on numerous factors, including the stage of tumor progression and the existence of patient co-morbidities, which may require a tailored management and treatment strategy (5). Local therapeutic strategies remain the gold standard for the treatment of early-stage HCC (5, 141, 142). Surgical tumor removal is the most effective approach currently available for patients with HCC (13). Patients with early-stage HCC may undergo a partial liver resection provided that there is only an isolated lesion(s), that their liver function is retained, and that no cirrhosis is observed (5, 20). HCC is often accompanied by cirrhosis, making liver transplants the most promising treatment option (116). However, due to the scarcity of donors, a liver transplant may not be immediately available for HCC patients (20, 116). According to Burley and Roth, bioartificial liver support is sometimes used to temporarily compensate for the damaged liver function while the patient awaits a liver transplant (124). However, due to the difficulty of hepatocyte isolation and *in vitro* culturing, bioartificial liver support is not only far inferior to normal liver function, but it is also not considered to be a permanent solution (124). Patients on the waiting lists for liver transplants often remain there for a significant length of time, sometimes more than a year, and risk being removed from the list if their conditions progress past the requirements of the Milan criteria (20, 143). The Milan criteria require tumor growth to fall within the following categories: (a) an isolated malignancy that is no greater than 5 cm, or (b) the presence of 2-3 tumors that are smaller than 3 cm, and (c) that the tumor is exclusively restricted to the liver tissue (20). To reduce or prevent the progression of HCC while patients await a liver donor, locoregional treatments (i.e., trans-arterial chemoembolization, radiofrequency ablation, and percutaneous ethanol injection), are often administered (20, 116, 144, 145). These locoregional treatments alone, or in combination, may offer a more promising option against early, unresectable HCC cases (146).

Chemotherapy is administered orally and serves as the only medical treatment option for HCC that does not include the physical infiltration of body tissues (146, 147). Sorafenib (Nexavar®) is to date the only chemotherapeutic drug approved by the Food and Drug Administration for exclusive use in the treatment of HCC (145, 146, 148). Sorafenib is a multiple kinase inhibitor that is administered as a first-line chemotherapeutic drug, followed by the administration of Regorafenib (Stivarga®) (5, 148). However, these drugs have their limitations, which include numerous adverse side effects such as dermal and cardiovascular complications (149). An increase in the development of Sorafenib drug resistance was also reported in patients in the advanced stages of HCC progression (5, 148, 149). The approved treatment strategy for different stages of HCC tumor progression, described as the Barcelona Clinic Liver Treatment Strategy (150).

Even with the medical advancements made to date toward the treatment of HCC, relapses, the prevention of metastasis and the recurrence of cancer continues to pose a significant clinical challenge (141). The risk of tumor recurrence continues to pose an obstacle in the treatment of HCC and has been observed in nearly 70% of all patients within the first five years of having received treatment (5, 13, 151). Advanced stage HCC is typically incurable, and this late-stage diagnosis worsens the prognosis for most HCC patients (5). Consequently, advanced HCC patients only have a 5% survival rate, therefore highlighting the importance of exploring alternative adjuvant therapies and therapeutic targets (14, 152).

The efficacy of current chemotherapeutics is further hampered by their inability to selectively target cancer cells without impacting the healthy cells (153). Single-molecule or drug approaches are less bioavailable due to their molecular structures and thus require the development of supporting compounds to increase their bioavailability and efficacy (154). Given the above limitations with current HCC therapeutics, the use of plant-derived extracts is increasingly being explored as an alternative adjuvant anti-cancer therapeutic (155, 156).

The use of Medicinal Plants in HCC Treatment

Plant-derived extracts often contain an array of compounds, making them more bioavailable and negating the need to develop specialized drug delivery systems (154). Numerous studies have identified and exploited naturally occurring bioactive compounds that have anti-cancer potential; often recommending these compounds as alternative adjuvant chemotherapeutic options to treat cancers, such as HCC (157, 158). Approximately 60% of all current anti-cancer therapeutics are derived from naturally sourced plant components (4). Exploiting naturally sourced medicinal plant materials known to have HCC anti-tumor properties may led to the discovery of adjuvant treatment solutions (155). Such adjuvant therapeutics could not only have significantly fewer side effects, but may also be more affordable for most patients in developing countries such as South African (155, 159).

Therefore, there is an increased research interest in the potential use of known medicinal plant-derived anti-tumor components as adjuvant therapeutic options in treating various cancers, including HCC (142, 156). For example, plant-derived bioactive compounds such as vinblastine, a chemotherapeutic drug that has been shown to increase HCC cell sensitivity to treatment, and irinotecan, a chemotherapeutic drug shown to cause DNA damage and induce apoptosis in cancerous cells such as HCC, have been derived from plants (160–162). Despite the rich potential of medicinal plants hold, medicinal plants that can be exploited for their anticancer properties remain largely understudied (158, 161).

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To this end, the *Moringa oleifera* (MO) tree is being increasingly investigated for its anti-cancer properties. MO contains bioactive compounds that have hepatoprotective properties such as the inhibitory potential observed against numerous viruses including HBV and the human immunodeficiency virus (HIV) (131, 163). Several studies have also reported on the therapeutic potential of MO in various HCC cells, including the $HepG_2$ cells (142, 153, 164, 165).

The use of *Moringa oleifera* in HCC Treatment

The MO tree, also referred to as the 'drumstick tree', belongs to the family Moringaceae (166, 167). MO is native to the sub-Himalayan Indian subcontinent; however, Indian migrants spread it to other tropical and subtropical parts of the world, including SA (153, 159, 167). This plant was initially consumed as a vegetable, but its numerous health benefits were soon recognized and are currently exploited in various traditional treatments and healthcare practices (142, 155). Different parts of the MO tree contain numerous phytochemicals (e.g., nutrients, amino acids, and carotenoids) proven to have anti-inflammatory, hepatoprotective, neuroprotective, antioxidant, immunity-enhancing, and anti-cancer properties (153, 155, 156, 166, 167). The dietary inclusion of MO has been reported to potentially aid in recovery after pathogen infection by scavenging free radicals which are produced by pathogens (163).

Despite all parts of the MO tree containing several bioactive compounds, the leaves have an abundance of polyphenols and polyflavonoids with antioxidant and anti-cancer properties (155, 168–170). The anti-cancer potential of leaf extracts has been mostly elaborated (171). The phytochemicals present in the MO leaf extracts have been found to display anti-cancer properties (159). Free radicals are formed under oxidative stress conditions, and the failure to control this production contributes to cancer development (159). According to a recent study by Wu and colleagues, MO leaf extracts have high concentrations of gallic acid, orientin quercetin, kaempferol, and catechin, all of which are potent antioxidants (168). The high concentration of antioxidants in MO leaf extracts is largely responsible for its potential to induce apoptosis within cancer cells (171). In a more recent study, Xu

and colleagues identified kaempferol 3-*0*-rutinoside, kaempferol 3-*0*-glucoside, quercetin 3-*0*-(6′-malonyl-glucoside), and quercetin derivatives as compounds within MO leaf extracts that exhibited the strongest antioxidant activity (172). Xu and colleagues further reported that these compounds showed strong free radical scavenging capacity as determined by both 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,20-azinobis-(3-ethylbenzthiazoline-6-sulfonic acid (ABTS) antioxidant determination assays (172). The antioxidants have been reported to activate apoptotic pathways in cancerous cells (171). MO leaves have the highest abundance of phenols and flavonoids which have the most antioxidant and free radical-scavenging activity (173) However, several studies also reported that MO may induce oxidative stress in cancerous cells. The bioactive compounds in MO can increase ROS levels in cancerous cells, leading to cellular damage and ultimate apoptosis (174, 175).

Sreelatha and colleagues reported on the anti-cancer potential of MO by showing dose-dependent morphological changes in human KB tumor cells, such as cytoplasmic membrane shrinkage, following MO leaf extract treatment (176). A study by Barhoi and colleagues also reported on the anti-cancer potential of MO leaf extract treatment (177). They showed a significant dose- and time-dependent decrease in Ehrlich ascites carcinoma cells (EAC) solid tumor volume and weight following MO aqueous leaf extract treatment (177). This study also concluded that MO leaf treatment increases the tissue restoration and survival of tumor-bearing rats (177). Furthermore, the normal physiology of the control rats was not affected by the MO leaf extract treatment, suggesting that its cytotoxicity is selective toward the cancerous cells (177). Supporting these findings, Patel and colleagues reported that the MO leaf extract was significantly less cytotoxic toward the VERO African green monkey's kidney cells (normal control cells), compared to various cancer cell lines, including the MCF-7 breast cancer cells, K562 leukemia cells, DU145 prostate cancer cells, and HCT15 colon cancer cells (178). In a study by Cordier and colleagues, a reduction in mitochondrial membrane potential in $HepG_2$ cells following MO aqueous leaf treatment was observed (174). The loss of mitochondrial membrane integrity results in an increase in ROS production due to the leakage of electrons (174). In a separate study, Al-Asmari and colleagues reported that MO leaf extracts had the highest anti-cancer potential, compared to extracts from other parts of the tree (179). This study showed a 90% decrease in breast cancer cells (MDA-MB-231) and colorectal cancer cells (HCT-8) motility following MO leaf extract treatment compared to a 50% decrease that followed MO bark extract treatment (179). In this study, it was also reported that MO leaf extract treatment had the highest apoptotic potential, as evidenced by a 19% increase in apoptotic cells compared to a 2% increase following MO bark extract treatment of MDA-MB-231, and HCT-8 cells (179).

Cancer therapeutic strategies (e.g., chemotherapeutics and radiation) aim to induce apoptosis in cancerous cells (180, 181). Bioactive compounds found in MO leaves have shown to be a viable adjuvant therapeutic option to current HCC chemotherapeutics (155). This is due to the proven ability of these bioactive compounds to induce apoptosis in various cancerous cells (such as breast cancer cells, colon cancer cells, and human liver cancer cells), and their overall efficacy in improving immune system responses (155). There are currently no clinical studies that have investigated the apoptotic capacity of MO on cancerous cells, however pre-clinical investigations have revealed the plants' ability to induce apoptosis in many cancer cells by controlling the activity of apoptotic cell markers (171). Studies have reported on MO's ability to induce apoptosis through the upregulation of oxidative stress in numerous cancerous cells including $HepG_2$ cells (169, 176, 182), and its ability to induce apoptosis through the action of its antioxidative phenols (171, 173, 183). Therefore, there is a need to understand the mechanism of MO-induced apoptosis toward the potential development of novel treatment strategies (184). Studies have revealed that MO leaf extract can induce apoptosis through numerous apoptosis-triggering events such as the up-regulation of apoptosis genes (e.g., p53 and Bcl-2 family of genes members), the induction of DNA fragmentation, and the suppression of angiogenesis and metastasis in different cancer cell lines (e.g., MCF-7 breast cancer cells, Caco2, and $HepG_2$ cells) (155, 185–187). It has also been reported that the phenols and other bioactive compounds within the MO plant activate pro-apoptotic proteins, initiating the apoptotic pathways (171).

Apoptosis

Apoptosis is programmed cell death that occurs in multicellular organisms (184). It is characterized by a series of morphological features, such as shrinking of the cell, nuclear shrinkage, fragmentation into membrane-bound apoptotic bodies, and phagocytosis by neighboring cells (188, 189). The shrinkage of the cell during apoptosis is a result of the collapse of intracellular structures due to the cleavage of their

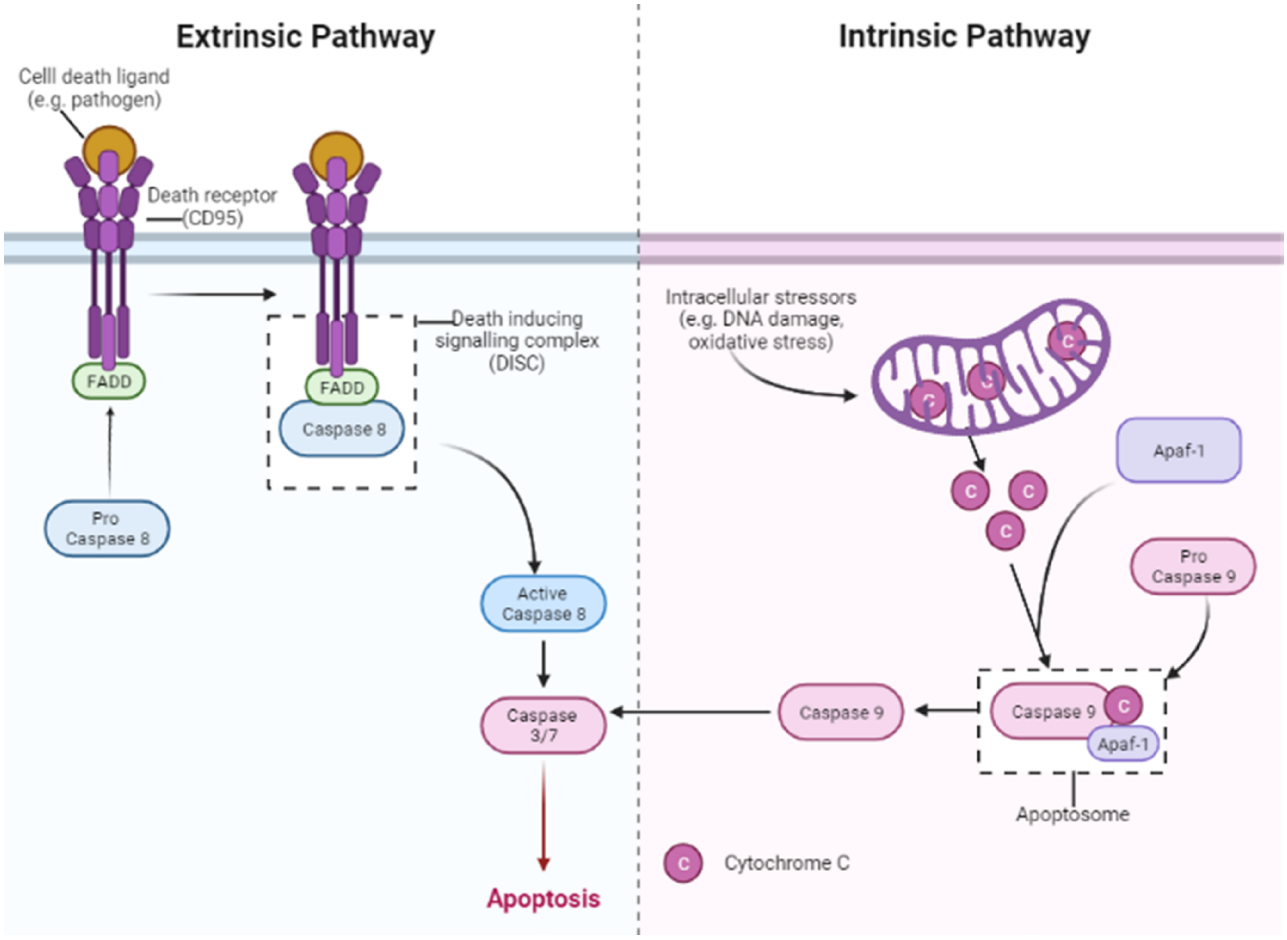

Figure 5. Schematic representation of caspase-mediated apoptotic pathway. Fas, transmembrane death receptor; FADD, Fas-associated protein with death domain (FADD); DISK, death inducing signaling complex; Apaf-1, apoptotic protease activating factor 1.

cytoskeleton, nuclear membrane disruption, and chromatin condensation (188, 189).

Apoptosis is a trigger-stimulated mechanism that regulates cellular self-destruction (190). This mechanism systematically eliminates damaged, irregular, or excess cells in response to stimuli or to maintain homeostasis (190). Apoptosis is governed by proteins referred to as caspases, which ensure that the cell is fragmented in a systemic manner (184, 188). Caspases are a family of cysteine proteases synthesized as catalytically inactive zymogens in cells and facilitate apoptosis activation (191–193). In mammalian cells, caspases are classified into two groups, the initiator and effector caspases (194). The initiator caspases (caspase-8 and –9) interact with upstream molecules that trigger the apoptotic process and the effector caspases (caspase-3 and –7) are activated by the upstream initiator caspases and are responsible for the downstream events such as the cleaving of intracellular components (194, 195). Initiator caspases occur in their inactive states within cells as monomeric zymogens (196). Activation of initiator caspases involves the recruitment of the caspase into a multi-protein activating complex, resulting in the dimerization of the caspase (196). The activating complex could either be intrinsic or extrinsic depending on the origin of the death stimulator (Figure 5) (184, 196).

The extrinsic pathway occurs *via* caspase-8 initiator caspase as follows: a compound with a cell death ligand such as cytokines, drugs, hormones, pathogens, and native activity compounds such as vitamin 3 and lycopene (a powerful antioxidant) bind with the transmembrane death receptor, CD95 (also named Fas, or apoptosis antigen 1) (184, 188, 189, 196). Following this ligation, Fas recruits a Fas-associated protein with death domain (FADD) which in turn recruit's caspase-8 (196). The resulting complex is known as a death-inducing signaling complex (DISK) (196, 197). Caspase-8 is activated within this DISK and the Fas/FADD/caspase-8 DISK activates caspase-3 (196).

The intrinsic pathway occurs *via* the caspase-9 initiator caspase as follows: intracellular stressors such as DNA damage, oxidative stress, ER stress, and growth factor withdrawal alter the permeability of the mitochondrial outer membrane (184, 188, 196). Mitochondrial outer membrane permeabilization results in the release of cytochrome c (184, 196, 198). Cytochrome c binds with apoptotic protease activating factor 1 (Apaf-1) within the cytosol, forming a complex that recruits caspase-9 to form an apoptosome, which is a large protein formed during apoptosis (195, 196). Caspase-9 is activated within the apoptosome and the cytochrome c/Apaf-1/caspase-9 apoptosome complex activates caspase-3(200,202).

Executioner caspases (caspase-3 and caspase-7) are dimers in their inactivated state (196). Caspase-8 and caspase-9 activate executioner caspases by breaking down the links between their domains (184, 196). Once activated, caspase-3 cleaves numerous intracellular components, resulting in apoptosis (Figure 5) (198). An increase in caspase activity indicates an increase in apoptosis (155, 194).

A recent study by Ahmed and colleagues revealed the anti-cancer effects of MO leaf extract as evidenced by a dose-dependent increase in caspase activity (155). Importantly, the disruption of apoptosis contributes to the progression of tumorigenesis (199). The upregulated proliferation of tumor cells is partly due to the avoidance of apoptosis of these transformed cells (171). These cells achieve this by having a reduction in caspase activity, aberrant pro-apoptotic and anti-apoptotic protein composition, and activity, p53 gene mutation and a loss of some death receptors (171).

The p53 gene has been shown to play a role in cellular stress protection, in the regulation of glycolysis, autophagic pathways, and cellular differentiation (200, 201). Under physiological conditions, the mouse double minute 2 homolog (MDM2) targets and degrades p53, thus resulting in low basal levels of this protein (202). However, stressors such as oncogene activation and DNA damage inhibit MDM2, thus upregulating the activation of p53 (202, 203). A variety of pro-apoptotic proteins are activated by p53, including proteins such as BH3 domain only pro-apoptotic proteins, death receptors and apoptosis execution factors, which participate in various steps of the apoptotic pathway (203, 204). The activation of BH3 (a pro-apoptotic member of the Bcl-2 protein family), results in mitochondrial membrane permeabilization, a key step in the intrinsic pathway (204). Mitochondrial membrane permeabilization is controlled by the Bcl-2 family proteins, and the activation of pro-apoptotic Bcl-2 proteins Bax and Bak result in the formation of pores on the mitochondrial membrane, ultimately resulting in the release of cytochrome c (204). The activation of p53 also suppresses the inhibition of pro-apoptotic Bcl-2 proteins through the multifunctional anti-apoptotic protein, Bcl-XL (204). The activation of p53 can also induce extrinsic apoptosis through its activation of death receptors Fas and Dr5 (204), and plays a pivotal role in carcinogenesis through its apoptotic action (203). Under stressful conditions such as DNA damage, the activation of p53 prevents the accumulation of gene mutations which may lead to carcinogenesis (203). The loss of apoptosis control has been found to promote the prolonged proliferation of transformed cells, providing more time for the accumulation of cellular mutations, and subsequently increasing tumor cell invasiveness and metastasis (199). The mutation of p53 is observed in many tumors including, HCC (200, 205). This mutation results in a loss of function, a key contributor to carcinogenesis (205). The mutation of p53 in HCC cells has been shown to be closely associated with recurrence and chemotherapeutic avoidance (205). The activation of p53, ultimately resulting in the induction of apoptosis within cancerous cells, is the desired outcome for many chemotherapeutic drugs (204, 206, 207).

Several studies have shown that MO leaf extract has cytotoxic effects against selective cancer cell lines, including the $HepG_2$ cells (155, 159, 164, 165). In a study by Fisall and colleagues, an increase in the activity of p53 and the expression of other pro-apoptotic proteins such as Bax was reported following MO leaf extract treatment exposure in MCF-7 cells (208). Similarly, another study reported a significant increase in p53, and CASP3 gene expression following MO leaf extract treatment of $HepG_2$ cells, ultimately resulting in apoptosis (209). CASP3 expression is directly correlated to the execution phase of apoptosis (209). MO leaf extract also suppressed the expression of anti-apoptotic genes Bcl-2 and matrix metalloproteinase 1 (MMP1) (209). MO leaves have a high abundance of antioxidants, and this has been shown to have an inhibitory effect on anti-apoptotic proteins (171), thus favoring apoptosis. The human $HepG_2$ cancer cell line is particularly hypersensitive to galactosamine, a hepatotoxicant phytochemical found in MO leaves (153, 210). Studies have also shown that MO leaf extract exerts a concentration-dependent reduction in the cell viability of $HepG_2$ cells (142, 153, 159, 165). Consistent with these findings, Abd-rabou and colleagues reported a 68% reduction in the human $HepG_2$ cancer cell viability following

MO leaf extract treatment (153). Additionally, this study revealed a dose-dependent decrease in the ATP levels in the $HepG_2$ cancer cells, accompanied by cell death, with the lowest ATP levels observed at the highest dosage of MO leaf extract treatment (153). However, it was not clear whether the cytotoxicity of the MO leaf extract was responsible for the ATP disruption, thus leading to apoptosis; or if it directly induced apoptosis, which in turn resulted in ATP disruption (153). A separate study revealed that MO leaf extract helps the liver to evade the effects of diethylnitrosamine – a compound that causes severe damage to the liver, often resulting in hepatocarcinogenesis, even in low concentrations (211, 212). Susanto and colleagues discovered that the MO leaf extract has a dose-dependent inhibitory effect on the transforming growth factor-beta (TGF-β) expression and its signaling pathway (212). TGF-β is a hallmark of liver fibrosis, which is an HCC risk factor (212).

Strong evidence suggests that autophagy-related pathways such as CMA may contribute to the survival of HCC cells in a time and tumor stage-dependent manner, as shown by the significant increase in LAMP2A expression in the HCC Huh7 liver cancer cell line (24, 74, 76). However, the effect of MO aqueous leaf extract on CMA activity remains unclear in the context of an *in vitro* model system, using the HCC $HepG_2$ cancer cell line. The therapeutic potential of CMA blockage has been demonstrated using small interfering RNA silencing of the LAMP2A gene in multiple cancer cell lines (24). Studies have shown that the blocking CMA, either through genetic manipulation or pharmacological inhibition, can lead to the accumulation of toxic proteins or induce cell death in cancer cells (24, 213–215). Small interfering RNA (siRNA) silencing of the LAMP2A gene, which encodes the LAMP2A protein, has been shown to effectively block CMA and inhibit tumor growth in multiple cancer cell lines (216–218). In addition to its potential as a cancer therapy, CMA inhibition has also been explored as a potential treatment for other diseases, including neurodegenerative disorders such as Alzheimer's disease (219, 220). However, more research is needed to fully understand the role of CMA, as well as to develop effective and safe CMA modulators for clinical use.

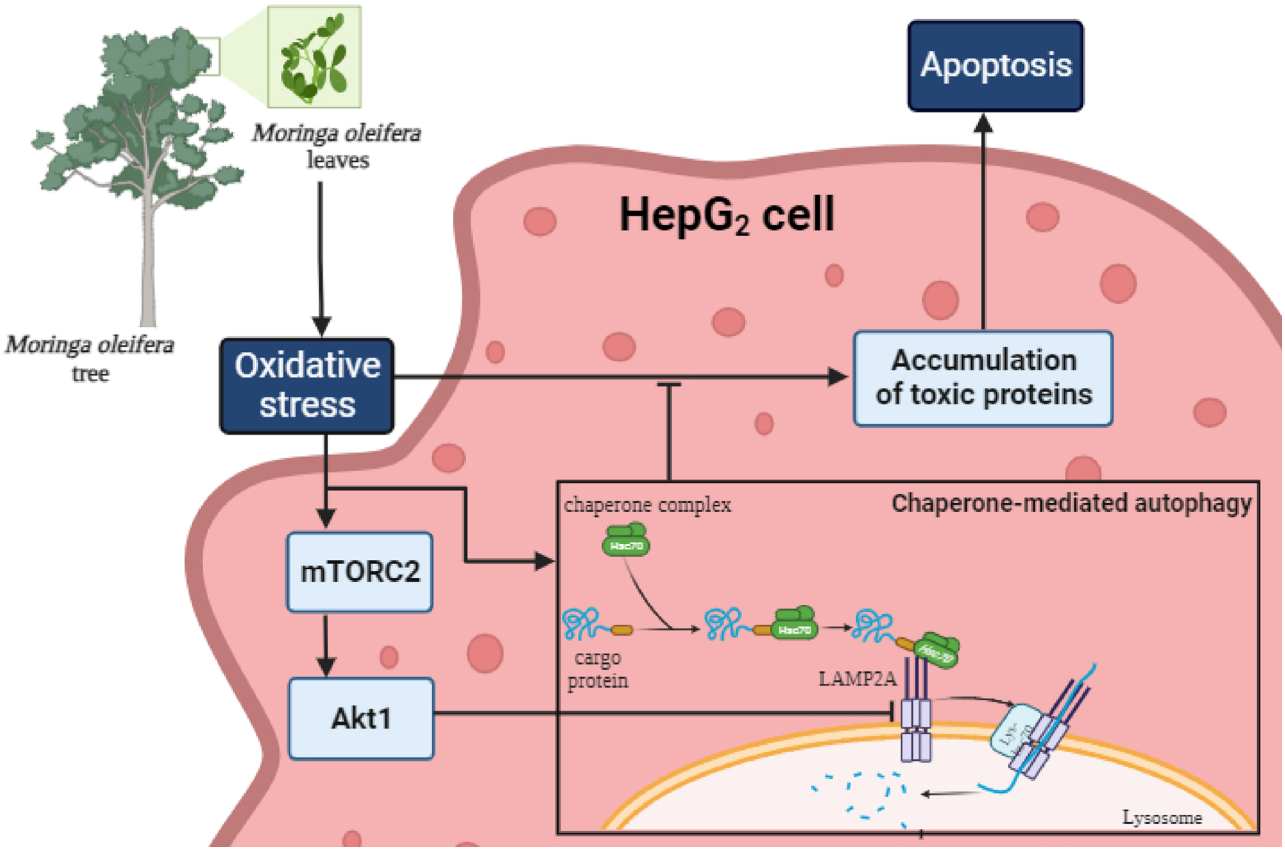

Figure 6. A proposed mechanism of interaction between *Moringa oleifera* and the chaperone-mediated autophagy pathway in $HepG_2$ cancer cells. Akt, Protein kinase B; HSC70, heat shock cognate 71 kDa protein; LAMP2A, lysosome-associated membrane protein type 2 A; Lys-hsc70, lysosomal variant of HSC70; mTORC2, mammalian target of rapamycin complex 2.

Future Perspectives and Recommendations

The effective use of conventional approaches in the long-term treatment of HCC remains an unmet clinical challenge. Research on the interaction between MO and CMA is still in its early stages. However, given the established oxidative stress effect of MO, and inducing effect of oxidative stress on CMA activity in cells, it is plausible to hypothesis that MO leave extract could potentially modulate CMA activity in cells. Given the potential health benefits of both MO and CMA modulation, future research may focus on elucidating the mechanisms underlying the interaction between MO and CMA, as well as on developing targeted interventions that can harness this interaction for therapeutic purposes. For example, researchers could explore the potential use of MO extracts as CMA modulators for the treatment of diseases such as cancer, neurodegenerative disorders, and metabolic diseases. However, additional research is warranted, to fully understand the effects of MO on CMA and its potential therapeutic applications in liver cancer cells, to favor apoptosis onset in these HCC cells. All these efforts would ultimately be aimed at attenuating the toxic side-effects of current cancer therapeutics through the use of adjuvant medicinal plant therapeutics, and ultimately, toward improved HCC patient prognosis and outcomes. The interaction between MO and CMA presents a novel avenue for therapeutic intervention in liver cancer cells. Researchers could explore the use of MO extracts as CMA modulators for the treatment of liver cancer and other related diseases. This approach could potentially enhance the effects of existing cancer therapies and lead to improved patient outcomes. While all intricacies of the complex biological processes involved are not fully captured, Figure 6 provides theoretical illustration of the proposed interaction between MO and CMA. When MO is used to treat cancerous cells, its high abundance of antioxidants induces oxidative stress within the cells. This oxidative stress triggers various cellular responses, including the activation of mTORC2, which subsequently activates Akt1. Akt1, when activated, can inhibits the CMA pathway, disrupting the cells' ability to remove damaged proteins. This disruption, combined with the oxidative stress-induced accumulation of toxic proteins, could potentially overwhelm the cellular stress response systems. As a result, the cells may experience a heightened level of cellular stress, ultimately leading to apoptosis. This dual effect, where MO-induced oxidative stress amplifies the impact of Akt1-mediated CMA inhibition, underscores a potential mechanism for bringing about cell death in cancerous cells treated with MO. notably, experimental validation would be needed to confirm the precise mechanisms and effects in this context.

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Disclosure Statement

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