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Review

Piperlongumine as anticancer agent: The story so far about killing many birds with one stone

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Abstract: Piperlongumine is a biologically and pharmacologically active constituent of the plant *Piper longum*. This compound is gradually gaining attention because of its ability to inhibit/prevent different cancers. Modern era of molecular oncology is incomplete without ground-breaking discoveries made in the field of cell signaling pathways. High-throughput technologies have considerably improved our understanding about wide ranging signal transduction cascades which play crucial role in cancer development and progression. It is exciting to note that piperlongumine has been shown to pleiotropically modulate different oncogenic signaling pathways. We partition this multi-component review into discrete sections and categorically summarize key findings related to excellent ability of piperlongumine to therapeutically target JAK-STAT, NF-kB and PI3K/AKT/mTOR pathways. We also set spotlight on regulation of TRAIL pathway and autophagy by piperlongumine in different cancers. On the basis of the current understanding of piperlongumine, molecular biologists and pharmacologists will develop the next generation of translational studies, which will prove to be helpful in improving the clinical outcome and getting a step closer to personalized medicine.

Key words: Cancer; Apoptosis; Signal Transduction Cascade; Piperlongumine.

Introduction of Piperlongumine

Because of rapidly developing resistance against therapeutic interventions, it is becoming increasingly essential to search for pharmacologically active molecules from natural sources (1,2). Research over decades has demystified multifactorial nature of cancer and we now know that intra- and inter-tumor heterogeneity, adaptative ability of evolving populations to temporally varying environments, greater motility and invasive potential, and highly plastic phenotypes, allow broader metabolic adaptability and increased capacity to rapidly shift to higher rates of proliferation and profound quiescence make cancer therapeutically challenging.

Natural products always occupy a prominent position as a source of new drugs, or as reference molecules, from which new drugs are discovered (3). In the *Piper* genus there are several pharmacologically active plants which have been tested for efficacy. Piperlongumine is also known as piplartine. It has increased the attention of researchers due to its ability to target different proteins in various cancers (4, 5). This compound has been described as an alkamide and can be found in several species of *Piper*. Its chemical structure has a phenylpropanoid substructure attached to a cyclic amide. Studies of its metabolism revealed the presence of various metabolites (6, 7). For example, in an analysis using CYP450 in vitro oxidation it was shown the formation of four metabolites: three products formed from the modification of the lactam, and one compound generated by demethylation of a methoxyl of the cinnamic moiety of piperlongumine (7). These metabolites may contribute to the antitumor activity of alkamide. In fact, several synthetic piperlongumine analogs have shown antitumor activity. In addition to the anticancer potential of this compound, its chemical structure has been an interesting prototype for the search for new drug candidates (8, 9). Keeping in view the need to identify natural products having maximum anticancer activity and ability to modulate multiple deregulated signaling pathways, we will focus specifically on anticancer activities of piperlongumine. There are some good reviews which comprehensively

describe chemistry and basic information associated with piperlongumine (10, 11, 12, 13). However, in this review we have set spotlight on anticancer activities of piperlongumine and we also identify the existing gaps which need to be bridged for an effective translation of laboratory findings to clinically effective therapeutics. In this review we systematically discuss deregulated signaling pathways targeted by piperlongumine. JAK-STAT pathway is a critical signaling cascade in cancer development and progression. Therefore therapeutic targeting of JAK-STAT pathway is necessary and we will critically evaluate most recent developments linked with targeting of JAK-STAT pathway by piperlongumine. We have also shed light on NF-kB signaling pathway and how it can be targeted to inhibit cancer. TRAIL mediated signaling is also very essential when it comes to the list of highly efficient agents for treatment/prevention of cancer. Therefore we tactfully analyze the outstanding questions which need extensive research.

Piperlongumine-Mediated Targeting of JAK-STAT Signaling

JAK (Janus Kinase)/STAT (Signal transducer and activator of Transcription) pathway has been shown to play vital role in cancer development and progression. Ligand-receptor interaction ignited rapid activation of receptor-associated JAKs which consequently phosphorylated STAT proteins. Functionally active phosphorylated STAT proteins moved into the nucleus and transcriptionally regulated expression of myriad of genes. There has been a renewed interest in identification of molecules having powerful activity against different STAT proteins to therapeutically target JAK-STAT signaling pathway. In this section we will be focusing on the premium activity of piperlongumine against different STAT proteins in various cancers.

B-cell acute lymphoblastic leukemia (B-ALL) exhibited constitutively active STAT1, STAT3 and STAT6. Piperlongumine inhibited STAT1, STAT3 and STAT6 in B-ALL cells (14) (shown in figure 1).

Multiple analogues of piperlongumine have been designed by replacing the cyclic amide of piperlongumine with aliphatic amides (15). Compound CG-06 was found to be strongly effective against prostate DU-145 cancer cells. Drug affinity responsive target stability (DARTS) provided evidence of physical binding of CG-06 directly to STAT3. CG-06 drastically reduced IL-6-driven STAT3 phosphorylation at 705th tyrosine in LNCaP and DU-145 cell lines (15) (shown in figure 1).

Drug resistance is a major stumbling block that confounds standardization of therapy. Therefore, it is necessary to search for the chemicals which can reverse drug resistance. Piperlongumine has been shown to reverse bortezomib resistance in multiple myeloma cells (16). Piperlongumine inhibited STAT3 activity by formation of a covalent linkage with a cysteine residue. More importantly intraperitoneal injections of piperlongumine markedly induced regression of tumors in mice subcutaneously injected with NCI-H929 cells (16). Piperlongumine also effectively inhibited tumor growth and development in mice xenografted with MDA-MB-468 breast cancer cells (17). Piperlongumine concentration-dependently reduced the phosphorylated levels of JAK1, JAK2 and STAT3 in gastric cancer cells (18) (shown in figure 1).

It seems clear that piperlongumine is highly effective against different STAT proteins and an efficient agent to interfere with deregulated JAK-STAT signaling in multiple caners.

NF-kB Signaling Pathway

Piperlongumine inhibited the proliferation of pancreatic cancer cells and potentiated the killing activity of gemcitabine (19). Piperlongumine inhibited the inducible and constitutive activation of NF-κB and suppressed NF-κB-driven target gene network. Furthermore, piperlongumine significantly induced regression of tumors and enhanced the efficacy of gemcitabine. Marked reductions in proliferation potential of cancer cells (PCNA and Ki-67), decreased micro-vessel density (CD31) and increased apoptotic rates were some of the prominent results noted in xenografted mice (19). Piperlongumine had previously been shown to bind directly to p65 subunit of NF-κB and consequently suppressed nuclear import of p65 (20). However, mutation of Cys(38) to Ser in p65 drastically interfered with ability of piperlongumine to inhibit nuclear accumulation of p65 in ABC-DLBCL cells (20).

Phosphorylation and degradation of IκBα is an essential step to trigger activation of NF-κB (21). Piperlongumine directly interacted with IKK (IκBα kinase) and inhibited its activity (shown in figure 1). Piperlongumine inhibited IKK through interaction with its 179th Cysteine residue (21). Furthermore, mutation of this residue abolished the activity of piperlongumine. NF-κB inhibition induced downregulation of proteins involved in survival (Bcl-2, Bcl-xL, c-IAP1, c-IAP2, survivin), proliferation (c-Myc, cyclin D1), invasion (ICAM-1, -9, CXCR4, VEGF) and inflammation (COX-2, IL6) (21).

Piperlongumine increased ROS, reduced glutathione, activated p38 MAPK and JNK, increased IκBα, and suppressed NFκB in LN229 glioblastoma cells (22). Piperlongumine mediated inhibitory effects on LN229 cells were completely abolished by the antioxidants. Use of JNK (SP600125) or p38 (SB203580) inhibitors significantly reduced the inhibitory effects of piperlongumine on migratory potential of LN229 glioblastoma cells (22).

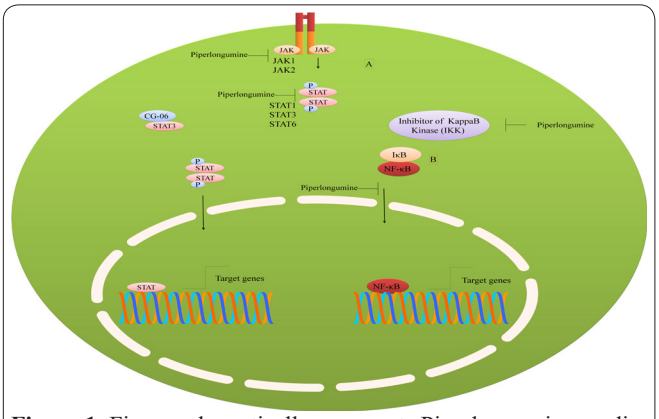

Figure 1. Figure schematically represents Piperlongumine mediated targeting of different pathways. (A) Piperlongumine effectively targeted JAK-STAT pathway. (B) Piperlongumine inhibited activation of NF-κB.

PI3K/AKT/mTOR Pathway and Autophagy: Push and pull

Physiologically, autophagy captures intracellular components in autophagosomes and delivers them to lysosomes for degradation and recycling. There has been much debate about diametrically opposed roles of autophagy in cancer. Autophagy has been shown to exert tumor suppressive effects through removal of damaged organelles, oncogenic proteins and toxic unfolded proteins. But on the contrary, autophagy can prove to be very dangerous because of its ability to fuel cancer through intracellular recycling for provision of substrates for metabolism and maintenance of functional pool of mitochondria.

Piperlongumine significantly reduced p-AKT, p70S6K1, 4E-BP1, Bcl-2, cyclin D1 and increased Bax and cytochrome-c in triple-negative BCa cells (23).

In this section we will summarize the available literature related to regulation of autophagy by piperlongumine and we devise a way forward for advantageous targeting of autophagy by tactful analysis of the context-dependent roles of autophagy and by capitalizing on modern approaches.

Series of ATG protein complexes orchestrated to form double membrane vesicles called autophagosomes that tactfully captured cytosolically distributed cargo (24, 25). Cargos can either be misfolded/damaged or highly abundant proteins or organelles that are ubiquitinated and identified by autophagy receptors such as SQSTM1/p62. It is important to mention that lipidated LC3-II molecules provide unique docking sites for specified cargo receptors, such as SQSTM1/p62. Different receptors have been shown to structurally bind to LC3 by specialized LC3-interacting region (LIR) for the recruitment of cargos for degradation. Fusion of lysosomes and autophagosomes was necessary for degradation of the cargo (24, 25).

Piperlongumine has been shown to effectively inhibit PI3K/AKT pathway to induce autophagy in cancer cells (26). Piperlongumine stimulated the expression of LC3-I in leukemic cells. Moreover, there was an increase in the levels of p38 MAPK and caspase-3. However, expectedly, piperlongumine blocked mTOR (mechanistic target of Rapamycin) and AKT (26). Piperlongumine evidently enhanced autophagy-associated proteins (Beclin-1, LC3B), and phosphorylation of JNK and p38 in BMMNCs (Bone marrow-derived mononuclear cells) obtained from the myeloid leukemia patients (27). However, pretreatment with SB203580 (p38 inhibitor) or SP600125 (JNK inhibitor) partly reversed piperlongumine-induced production of the ROS and killing activity (27).

Piperlongumine has been shown to reverse drug resistance mainly though downregulation of MRP1 (Multidrug resistance-associated protein-1), Top-II, GST-π, survivin, Bcl-2, CDK1 (cyclin dependent kinase-1), ABCB1 (ATP-binding cassette, Subfamily B, member-1) and ABCG1 (28). Furthermore, piperlongumine blocked PI3K/AKT signaling axis. Data clearly suggested that piperlongumine reversed the drug resistance of retinoblastoma cells SO-Rb50/CBP and HXO-RB44/VCR (28).

Piperlongumine-Mediated Activation of Autophagy Protected Cancer Cells from Apoptosis

Piperlongumine induced autophagy in docetaxel-resistant A549/DTX cells (29). Excitingly, treatment of A549/DTX cells with autophagy-specific inhibitors (3-methyladenine) or genetic inhibition of Beclin1 and ATG5 enhanced piperlongumine-mediated apoptosis. It was clear that piperlongumine-mediated activation of autophagy protected drug-resistant cancer cells from apoptosis. Therefore, inhibition of autophagy was essential to enhance piperlongumine-mediated apoptosis. Besides, piperlongumine inhibited PI3K/AKT/mTOR pathway (29).

Piperlongumine-Mediated Activation of Autophagy Enhanced Killing Effects

Suppression of piperlongumine-mediated cell death was observed in HeLa cells treated with 3-methyladenine, an autophagy inhibitor (30). Use of N-acetyl-cysteine (NAC) markedly reduced piperlongumine-induced autophagy and cell death which clearly suggested instrumental role of intracellular ROS in piperlongumine-induced autophagy. Piperlongumine also effectively stimulated p38 through ROS-induced stress response and emerging evidence has substantiated central role of p38 signaling in enhancing inhibitory effects of piperlongumine. Piperlongumine mediated autophagy induction as abrogated in dominant-negative p38 expressing cancer cells (30). PRIMA-1 (p53-reactivation and induction of massive apoptosis-1) and its methylated version PRIMA-1Met (APR-246) effectively re-established DNA-binding activity of p53 mutants and restored the functionalities of wild-type p53 (31). Piperlongumine worked synergistically with APR-246 and selectively induced apoptosis and autophagy in HNSCC cells. Combinatorial treatment potently induced conversion of LC3-I to LC3-II which provided evidence of induction of autophagy (31). Piperlongumine-induced inactivation of AKT/mTORC1 signaling promoted autophagy as evidenced by an increase in the levels of LC3-II (32).

TRAIL Signaling: Piperlongumine Joins Hand with TRAIL

It is becoming progressively more understandable that cancer cells develop resistance against apoptosis. Scientific breakthroughs have identified multiple mechanisms which underlie loss of apoptosis. Downregulation/inhibition of pro-apoptotic proteins, Overexpression of anti-apoptotic proteins, inactivation of intrinsic and extrinsic pathways and downregulation of death receptors on surface of cancer cells are some of the deeply studied processes. It was in mid 90s when TRAIL started to gain limelight because of its ability to kill cancer cells. Discovery of TRAIL revolutionized the field of molecular oncology and researchers were confident worldwide that they have finally identified the molecule which can be very effective in the treatment of cancer. However, contemporary studies identified various proteins which severely impaired TRAIL-induced apoptosis in different cancer cell lines. TRAIL (TNF-related apoptosis-inducing ligand) has been shown to transduce

the signals intracellularly though death receptors (DR4 and DR5). However, downregulation of death receptors is a frequently reported mechanism in TRAIL-resistant cancer cell lines. There has been an exponential increase in the number of natural and synthetic products reported to stimulate the expression of death receptors.

Piperlongumine concentration dependently induced apoptotic cell death and suppressed DNA binding activities of NF-κB in NSCLC cells (33). Docking models and pull-down assays demonstrated that piperlongumine directly interacted with DNA binding site of p50 subunit of NF-κB. Furthermore, co-treatment with NF-κB inhibitor substantially enhanced piperlongumine-mediated inhibitory effects on cell growth and activation of DR4 and Fas. Piperlongumine-triggered growth inhibitory effects were impaired in p50 mutant expressing A549 and NCI-H460 NSCLC cells. Likewise piperlongumine was unable to significantly stimulate expression of Fas and DR4 in p50 mutant expressing A549 and NCI-H460 NSCLC cells (33). Piperlongumine stimulated C/EBP homologous protein (CHOP), which resulted in upregulation of DR5 (34). Pretreatment with the ROS scavenger abolished piperlongumine -induced upregulation of CHOP and its target genes which clearly pinpointed towards essential role of ROS in piperlongumine-induced CHOP activation. Downregulation of CHOP with siRNA efficiently attenuated piperlongumine-induced apoptosis (34). Similarly, piperlongumine time- and concentration- dependently stimulated DR5 expression in DU145 and MDA-MB-231 cells (35). Mitogen-activated protein kinases (MAPKs) have also been reportedly involved in stimulation of death receptors. Treatment of MDA-MB-231 or DU145 cells with SP600125 (JNK inhibitor) suppressed piperlongumine-induced DR5 stimulation. Correspondingly, piperlongumine-induced DR5 upregulation was abolished when cells were pretreated with SB202190 (p38 inhibitor) (35).

Piperlongumine Mediated Targeting of Specificity Proteins

Piperlongumine was noted to significantly downregulate SP (Specificity proteins) in cancer cells (36). SP1, SP3, SP4 and SP-regulated genes were notably reduced in different cancer cell lines treated with piperlongumine. It had previously been convincingly revealed by O'Hagan and coworkers that hydrogen peroxide induced genome-wide shiftings of chromatin-modifying protein complexes from non-GC-rich to GC-rich promoters which consequently resulted in downregulation of c-Myc (37). Induction of ROS by piperlongumine exerted repressive effects on cMyc-regulated miR-27a, miR-17 and miR-20 and simultaneously upregulated ZBTB10 and ZBTB4. Piperlongumine induced an increase in gene inactivation mark H3K27 and slightly reduced the activation marks H3K4me3 and H4K16Ac in promoter regions of c-Myc and SP1 (36).

Piperlongumine Mediated Downregulation of PRC1: Role of P53

PRC1 (Protein regulator of cytokinesis-1) was originally identified as a mid-zone-associated protein required for cytokinesis. CDK1-driven phosphorylation of PRC1 turned it into functionally inactive and monomeric form (38). It was dephosphorylated and consequently interacted with a kinesin motor, KIF4 during the metaphase–anaphase transition. Dephosphorylated PRC1 played contributory role in the bundling of the anti-parallel inter-digitating microtubules for establishment of a mid-zone necessary for cytokinesis (38). p53 has previously been shown to transcriptionally downregulate PRC1 in cancer cells. Surprisingly, p53 levels were found to be considerably enhanced in AGS cells treated with piperlongumine. Whereas, piperlongumine mediated suppression of PRC1 was impaired partly in p53 depleted AGS cells. Mitotic-related genes including, BUB1B, KIF4A, NEK2, AURKB, MELK, NUSAP1 and TOP2A were notably reduced in PRC1 depleted AGS cancer cells (38).

F-Actin: Target of Piperlongumine

During carcinogenesis, cancer cells have the ability to migrate either as single cells or collectively as a group in a lamellipodium-based migration mode. Therefore in these conditions, there is an extension of cell membrane and lamellipodia are driven through polymerization of F-actin.

Piperlongumine was noted to reduce formation of lamellipodia and decreased F-actin structural assembly in bladder cancer cells (39).

Rapidly evolving knowledge related to metastasis has helped us to unravel various regulators which positively and negatively regulate formation of F-actin. Therefore efficient targeting of F-actin and its regulators will prove to be therapeutically effective. It was exiting to note that circular RNAs secreted by pancreatic cancer cells were taken up by endothelial cells (40). These endothelial cells demonstrated markedly enhanced metastasizing potential. Mechanistically it was shown that circular RNA increased F-actin expression, focal adhesion enhanced and promoted invasion and metastasis (40). CRYBG3, long non-coding RNA exerted repressive effects on formation of F-actin by inhibiting its assembly instead of promoting its disassembly (41). CRYBG3 interacted with G-actin and strictly prevented its conversion to F-actin (41).

Nanotechnological Approaches to Increase the Delivery of Piperlongumine

Piperlongumine was encapsulated into biodegradable polymeric micelles for analysis of efficacy (42). Piperlongumine micelles proficiently inhibited tumor growth in a subcutaneous CT-26 murine tumor model (42). Paclitaxel and piperlongumine were formulated into poly lactic-co-glycolic acid and D-α-tocopheryl polyethylene glycol succinate by organic solvent evaporation methodology (43). Paclitaxel and piperlongumine encapsulated nanoparticles (NPs) worked with effective synergy and combinatorially inhibited tumor growth in mice xenografted with HepG2 cells (43). Methoxy poly(ethylene glycol)-grafted chitosan (ChitoPEG) was cross-linked using selenocystine-acetyl histidine conjugates (ChitoPEGse) for delivery of stimuli-responsive piperlongumine (44). Piperlongumine was released from redox- and pH-responsive NPs in a stimuli-sen-

sitive manner and inhibited pulmonary metastases of colorectal cancer cells (44).

Conclusion

Piperlongumine has remarkable potential to inhibit oncogenic signaling pathways. Rapidly growing wealth of information has demystified tremendous ability of piperlongumine to target JAK-STAT pathway ad restore apoptosis in TRAIL-resistant cancer cells. Furthermore, piperlongumine has also been noted to inhibit PI3K/AKT/mTOR pathway in cancer cells. Importantly, NF-kB pathway was efficiently blocked by piperlongumine and future studies must converge on increasing the efficacy of TRAIL-based therapeutics. Entry of TRAIL-based therapies into various phases of clinical trials substantiated excellent killing activity of TRAIL and agonistic antibodies. However, certain hints have emerged which highlight activation of NF-kB pathway in TRAIL-treated cancer cells. Therefore, combinatorial strategies will consisting of TRAIL and piperlongumine will be helpful in shutting down NF-kB pathway and maximizing TRAIL-induced apoptosis.

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