Review

Molecular Cancer Therapeutics

Maternal Embryonic Leucine Zipper Kinase: Key Kinase for Stem Cell Phenotype in Glioma and Other Cancers

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Abstract

Maternal embryonic leucine zipper kinase (MELK) is a member of the snf1/AMPK family of protein serine/threonine kinases that has recently gained significant attention in the stem cell and cancer biology field. Recent studies suggest that activation of this kinase is tightly associated with extended survival and accelerated proliferation of cancer stem cells (CSC) in various organs. Overexpression of MELK has been noted in various cancers, including colon, breast, ovaries, pancreas, prostate, and brain, making the inhibition of MELK an attractive therapeutic strategy for a variety of cancers. In the experimental cancer models, depletion of MELK by RNA interference or small molecule inhibitors induces apoptotic cell death of CSCs derived from glioblastoma multiforme and breast cancer, both *in vitro* and *in vivo*. Mechanism of action of MELK includes, yet may not be restricted to, direct binding and activation of the oncogenic transcription factors c-JUN and FOXM1 in cancer cells but not in the normal counterparts. Following these preclinical studies, the phase I clinical trial for advanced cancers with OTSSP167 started in 2013, as the first-in-class MELK inhibitor. This review summarizes the current molecular understanding of MELK and the recent preclinical studies about MELK as a cancer therapeutic target. *Mol Cancer Ther; 13(6); 1393–8. ©2014 AACR.*

Introduction

Maternal embryonic leucine zipper kinase (MELK) is a member of the snf1/AMPK family of protein serine/threonine kinases. MELK was initially identified from analysis of cDNA libraries as a maternally derived gene that is active in the unfertilized egg and pre-implantation embryo in mice (1). Initial studies that characterized MELK expression in mouse ontogeny demonstrated that MELK mRNA expression is restricted to extraembryonic chorionic tissue. Later, MELK mRNA was more broadly observed in epithelial cells of the limbs, tail, eyes, and nose at regions of mesenchymal–epithelial tissue interactions. These data suggest a role of MELK in embryonic spatial patterning and organogenesis. In addition, MELK was initially implicated in the cell cycle. In particular, MELK mRNA levels are elevated at mitosis (2). MELK is likely required for mitotic progression, because MELK phosphorylates CDC25B—a protein phosphatase that activates CDK1 and subsequently promotes entry into mitosis. In fact, MELK protein strongly colocalizes with the key mitotic proteins, including cyclin A, cyclin B, and CDK4.

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doi: 10.1158/1535-7163.MCT-13-0764

As a result of these data, the observation that numerous cancers have elevated expression of MELK was not surprising. Thus far, significantly higher levels of MELK have been demonstrated in human cancers of the colon, breast, ovaries, pancreas, prostate, and brain (glioblastoma multiforme, GBM) compared with normal cells (3, 4). Notably, a large-scale meta-analysis of microarray data identified MELK as a consistently expressed gene in the transcriptional profiles of undifferentiated cancers (5). Indeed, studies have illustrated a correlation between MELK expression and tumor malignancy grade for astrocytoma, breast cancer, and prostate cancer, as well as radiation and chemoresistance in colorectal cancer (4, 6–8). In addition, MELK expression is inversely correlated with survival periods of patients with multiple cancer types (9, 10). Taken together, MELK plays a key role in survival and proliferation of undifferentiated cancer cells. Furthermore, several recent studies have implicated the critical role of MELK in cancer stem cells (CSC). CSCs are defined as a subpopulation of cells with prominent tumor-initiating ability. These cells can give rise to a diverse array of tumor cells in response to intra- and intercellular signals and microenvironment. Therapy-resistant phenotype of CSCs highlights the significance of molecular characterization of these tumor cells. Thus, inhibition of MELK is likely an attractive molecular target for cancer therapy.

Discovery of structural moieties specific to MELK is critical for development of targeted MELK inhibitors, but has proved to be difficult. In fact, the structure and signaling pathways of MELK are still being researched. Recent findings about MELK's structure have uncovered a ubiquitin-associated domain, and an activation segment

with a disulfide bond (11, 12). Indeed additional studies are necessary to understand the complex structure and signaling cascade involving MELK. Such discoveries will assist in the development of MELK-specific inhibitors. In this review, we discuss key studies that have delineated cancer-specific MELK signaling, in hopes of invigorating interest toward targeting MELK in the clinical arena. We will also highlight existing studies of *in vitro* and *in vivo* targeting of MELK and summarize potential strategies to indirectly target MELK activity through blocking its interactions with other oncogenic signaling pathways.

Targeting MELK

RNA interference–mediated MELK-targeting strategies

Depletion of MELK with RNA interference has been utilized extensively in various experimental models to study the role of MELK in normal and cancer cells. In most of the studies, gene specific elimination of MELK was validated with multiple siRNA (or shRNA) constructs. Choi and colleagues demonstrated that siRNA-mediated depletion of MELK reverses therapy resistance and increase the susceptibility of colorectal cancer cells to radiation and 5-fluorouracil–induced cell death (8). Thus far, the effects of RNA-mediated MELK knockdown have been most extensively studied in human GBM (WHO grade IV glioma). Both *in vitro* and *in vivo* experiments using human GBM samples have been conducted. *In vivo* studies utilizing human CSCs are frequently done using xenograft mice models. This approach allows for quantitative measurement of malignant invasion and qualitative observation of disease progression. However, it is not useful for studying tumor invasion in humans.

It was found that MELK knockdown by siRNA or shRNA induced apoptosis of human CSCs in GBM (glioma stem cells, GSC) both *in vitro* and *in vivo*. MELK knockdown by shRNA also reduced tumorsphere formation—a surrogate assay for CSC phenotype—in MMTV-Wnt1 mammary tumor cells (13).

One concern with MELK inhibition is the effect on normal neural stem cells. Data have shown greater abrogation of cell proliferation and induction of apoptosis in MELK siRNA (or shRNA) treated GSCs than neural stem cells. Marie and colleagues also demonstrated that treatment with MELK siRNA diminished proliferation and increased apoptosis of glioma cell lines *in vitro* (6). Preferential induction of apoptotic cell death by MELK knockdown has raised a possibility that MELK-targeted therapies for cancer patients may result in few or no side-effects to normal organs.

Small molecule inhibitors of MELK

Although the elevation of MELK expression has been demonstrated in numerous cancers, no oncogenic mutations in the MELK gene have been identified to date. The latest genome-wide gene-sequencing analyses, made available by The Cancer Genome Atlas (National Institutes of Health; cancergenome.nih.gov), indicate that MELK seems to be overexpressed but not mutated in cancers. Perhaps complicating matters further, studies of MELK signaling in normal cells demonstrate a large and seemingly disjointed set of biologic substrates whose activation leads to diverse cellular processes such as cell-cycle progression and growth, cell migration, and DNA damage repair (2, 4, 14–17). Therefore, a small molecule compound that selectively inhibits MELK kinase activity may risk unwanted off-target effects in both normal and cancer cells. In the past year, we have published two studies demonstrating novel cancer-specific activation of c-JUN and FOXM1 signaling via MELK, two transcription factors with strong oncogenic potential (14, 18). Our findings suggest that MELK overexpression may result in abnormal protein–protein interactions that promote cancer progression. Identifying these uniquely cancerous MELK-signaling cascades affords potential to design therapeutics with greater specificity to cancer than would be afforded by MELK kinase inhibitors. To illustrate this, in the following section we will briefly discuss our findings from these studies and demonstrate that targeting MELK interactions with c-JUN and/or FOXM1, rather than MELK itself, may generate novel chemotherapeutic agents inhibiting cancer-specific MELK signaling.

Siomycin A and MELK signaling in GBM and prostate cancers

Siomycin A, a thiazole antibiotic, was initially identified as a FOXM1 inhibitor that acted by reducing transcriptional activity and destabilizing the FOXM1 protein (19–21). Subsequently, as part of a larger screening study, we found that Siomycin A diminished MELK mRNA levels in human and mouse GBM cells (15). In addition, Siomycin A treatment targeted the GSC population within the tumor and diminished their *in vitro* self-renewal, invasion, and resistance to apoptosis. Siomycin A treatment prevented mitotic entry and arrested treated cells in the G_2–M transition, a phenotype that has been previously observed after siRNA-mediated MELK silencing. Another study by Kuner exhibited that Siomycin A treatment also induces mitotic cell-cycle arrest in a prostate cancer cell line (4).

Through further experimentation with siomycin A, we recently elucidated MELK signaling pathways unique to GSCs and not present in somatic cells. Specifically, we demonstrated that MELK binds and phosphorylates FOXM1 at serine residues that, in turn, are essential for MELK kinase activity (14). This interaction is important, as FOXM1 is a major transcription factor regulating a diverse set of genes that are essential for mitotic cell progression in cancers (e.g., Aurora A/B, Survivin, and CDC25B; refs. 22 and 23). FOXM1 is also required for a variety of cancer cell phenotypes, including cell proliferation, mitotic progression, DNA damage repair, angiogenesis, and suppression of apoptosis (24–27). Siomycin A treatment significantly decreased the proportions of FOXM1(+), MELK(+) cells in GBM tumorspheres in a dose-dependent manner, suggesting that siomycin A may abrogate cancer-specific MELK signaling through

disruption of MELK-driven FOXM1 transcriptional activity. This specificity reduces the possibility of common toxicities related to mitotic kinase inhibition—impaired cycling in bone marrow cells and functional disruption in neuronal cells (28). Interestingly, temozolomide, the current first-line chemotherapeutic agent for GBM, paradoxically increases MELK and FOXM1 expression, further supporting the hypothesis that targeting MELK in combination with temozolomide may attenuate GBM cell growth in patients (14).

Collectively, although Siomycin A continues to be utilized experimentally in studying FOXM1 inhibition, there is now data supporting Siomycin A activity against MELK signaling in cancer cells. Future studies should investigate whether MELK inhibition by Siomycin A occurs in other cancers. Nonetheless, Siomycin A may not be a practical compound for future clinical use because of its relatively large molecular weight and complicated molecular structure, as well as lack of penetration of the blood–brain barrier. Although Siomycin A itself may have limited clinical utility, its preclinical use has enabled and will continue to clarify the molecular signaling events associated with MELK and potential therapeutic outcome in the experimental settings for a variety of cancer models. These issues aside, our recent finding of MELK-driven activation of FOXM1 activity in GSCs provides a basis for merging existing theories on the mechanism of action of Siomycin A against FOXM1 and MELK. It is yet to be seen whether this unique oncogenic complex of FOXM1 and MELK exists in other cancers as well.

c-JUN and MELK activity

Our group also began to investigate c-JUN, a downstream oncogenic transcription factor of JNK signaling. We found that circulating basic fibroblast growth factor (bFGF) produced by intratumoral vascular endothelial cells induced MELK expression in GBM cells. Further experiments with inhibitors of the bFGF-driven signals showed that JNK2 regulates MELK protein levels in GBM cells (18). Interestingly, treatment with the JNK2 inhibitor JNKiII specifically reduced MELK protein levels within the nucleus but not the cytoplasm in a cancer-specific manner. Endogenous MELK was bound to c-JUN within nuclei, thereby forming a protein complex unique to GSCs, yet undetectable in normal cells. In GBM, alteration of p53 signaling is observed in as many as 87% of tumor samples (29, 30). We found that the expression of p53 exhibited an inverse correlation with MELK expression: MELK silencing increased p53 expression and p53 inhibition increased MELK expression. In addition, we found that radiation of GSCs upregulated MELK mRNA and protein levels. Taken together with previous studies demonstrating p53 suppression by c-JUN, these data suggested that JNK-driven MELK/c-JUN signaling suppressed apoptosis and promoted survival of GSCs but not normal cells (31). The effect of MELK signaling on p53-intact samples of GBM is yet to be seen, but will provide additional interesting data on the precise mechanism and role of MELK in GSC signaling.

Because of the cancer-specific interactions between MELK and c-Jun, small molecule drugs (SMD) that target MELK-mediated c-JUN signaling could be effective on controlling cancer growth in patients. The only potential candidate molecule from our study was JNKiII, also known as SP600125, which was one of the earliest SMDs shown to inhibit JNKs (32). SP600125 has been shown to induce tumor regression through JNK inhibition, although it has too broad an array of downstream events for use as a clinical agent (33, 34). Thus, although JNKiII is useful as a tool compound, further drug discovery studies will be needed to identify analogs with fewer downstream targets. These compounds could become novel therapeutics with clinical utility.

Aurora kinases and MELK

Forced expression of the kinase dead mutant of MELK (D150A) significantly diminished the nuclear interaction between MELK and c-JUN, establishing that disrupting the kinase activity of MELK may be a viable means of abrogating MELK/c-JUN–derived signaling. Therefore, in this section, we shall discuss recently reported SMDs that have been shown to preclude MELK kinase activity and diminish the growth of human cancer cells.

Members of the Aurora kinase family, notably Aurora A and B, have garnered increasing attention in recent years as potential targets for SMD to treat cancers. In mammalian cells, they have been shown to maintain the integrity of mitosis, including chromosome condensation, mitotic spindle assembly, and successful cytokinesis (35–37). The search for Aurora kinase inhibitors led us to a novel molecule of the benzo[e]pyridoindole family, called compound 1 (C1). In addition to its inhibitory effects on Aurora B (IC_{50} = 310 nmol/L), C1 potently inhibited MELK kinase activity (IC_{50} = 42 nmol/L; in editing for press). Similar to the phenotypes by the genetic knockdown of MELK and the pharmacologic inhibition by Siomycin A, treatment of patient-derived GBM cells with C1 exhibited selective elimination of GSCs through induction of mitotic arrest and catastrophe and also showed *in vivo* suppression of tumor growth in xenografted mouse models of GBM.

Because C1 diminished expression and kinase activity of both Aurora B and MELK, it should be considered as a multikinase inhibitor. As with any other drugs with multiple targets, the combinatorial activities may either be advantageous or result in undesirable off-target effects (38–40). Until further research clearly elucidates interactions between MELK and Aurora B in cancer signaling, however, it is difficult to determine whether the dual inhibitory properties of C1 are beneficial or toxic. Multikinase inhibition may, in fact, be a desirable property in the design of a cancer drug.

Many of the current U.S. Food and Drug Administration–approved SMDs exhibit multikinase inhibition and demonstrate potent activity against certain cancers. For

example, sorafenib inhibits extracellular VEGF and PDGF receptor signaling as well as intracellular Raf/Mek/Erk (MAPK) cascades, and is clinically approved to treat advanced hepatocellular and renal cell carcinomas (41–44). Quite recently, crizotinib, a dual tyrosine kinase inhibitor of ALK and c-MET, was approved to treat non–small cell lung cancer and is also under exploration for treatment of anaplastic large cell lymphoma (45, 46). Finally, danusertib is a SMD directed against Aurora kinases, but has not been evaluated for off-target activity against MELK (47, 48). Given the similarity of the protein structures between MELK and Aurora kinases, danusertib can possibly possess some inhibitory effect on MELK kinase activity with clinical value.

OTSSP167 targets MELK phosphorylation of novel substrates

Recently, Chung and colleagues reported the development of a novel quinolone-based compound OTSSP167, which potently inhibits MELK kinase activity (16). Of note, OTSSP167 represents the most potent *in vitro* MELK inhibitor in the literature thus far (IC_{50} = 0.41 nmol/L). OTSSP167 treatment significantly diminished MELK phosphorylation of two proteins, debrin-like (DBNL) and proteasome α subunit 1 (PSMA1), both of which had not been previously known as MELK substrates. Further experimentation demonstrated novel roles of MELK phosphorylation of DBNL in promoting tumor cell invasion and of PSMA1 in maintaining stem cell properties in lung, breast, and prostate cancer cells.

OTSSP167 demonstrated high target specificity, minimal toxicity, and relative ease of administration. Treatment of xenografted mouse tumor models with OTSSP167 showed diminished growth of tumors derived from MDA-MB-231 (triple-negative breast cancer), A549 (lung cancer), DU145 (prostate cancer), and MIAPaCa-2 (pancreatic cancer) cell lines, all of which were shown to express high levels of MELK. Furthermore, OTSSP167 treatment did not affect growth of xenografted tumors in mice derived from MELK(-) PC-14 human bladder cancer cells, indicating the high specificity of OTSSP167 against MELK. In addition to high target specificity, the authors demonstrated significant oral bioavailability of OTSSP167 by showing similar potency of tumor growth inhibition in xenografted mouse models treated either intravenously or orally with comparable tumor growth inhibition (TGI) of 73% and 72%, respectively. Similar results were observed with oral OTSSP167 treatment of lung, prostate, and pancreatic cancer xenografts. Considering that treated mice exhibited little to no loss of body weight, OTSSP167 may have a potentially favorable low-toxicity profile. As the most potent MELK inhibitor discovered thus far and with demonstrated *in vitro* and *in vivo* efficacy and safety, OTSSP167 currently represents the best option for testing a MELK inhibiting SMD in patients with human cancer.

Following these positive preclinical data, a phase I human clinical trial with OTSSP167 was started in 2013 and is currently recruiting for patients with locally advanced or metastatic solid tumors, or for patients whose tumors are refractory to standard therapy or for which no standard therapy is available. Results of the initial dose escalation tests will be disclosed later in 2014.

In conclusion, a growing body of evidence for significant roles of MELK in cancer signaling strongly encourages development of novel MELK-targeted therapeutics for clinical application. Many favorable effects have been seen in the preclinical studies targeting MELK in a variety of cancers. Patients with cancers that have high levels of MELK expression may benefit from therapies inhibiting MELK. We hope that inhibition of MELK in human trials parallels the effects observed in preclinical experiments: notably, a reversal of tumor resistance to therapies, increased susceptibility of tumors to radiation, diminished proliferation and increased apoptosis of tumor cells, diminished self-renewal, and mitotic cell arrest. As demonstrated by Siomycin A and SP600125, discoveries of novel MELK signaling pathways concurrently allow for the testing of current experimental SMDs for the purposes of developing future drug candidates that selectively and potently target MELK. Thus far, these early studies have led to the identification of C1 and OTSSP167 as candidate drugs for MELK selective targeting. Data from these studies demonstrate that both use of previously identified

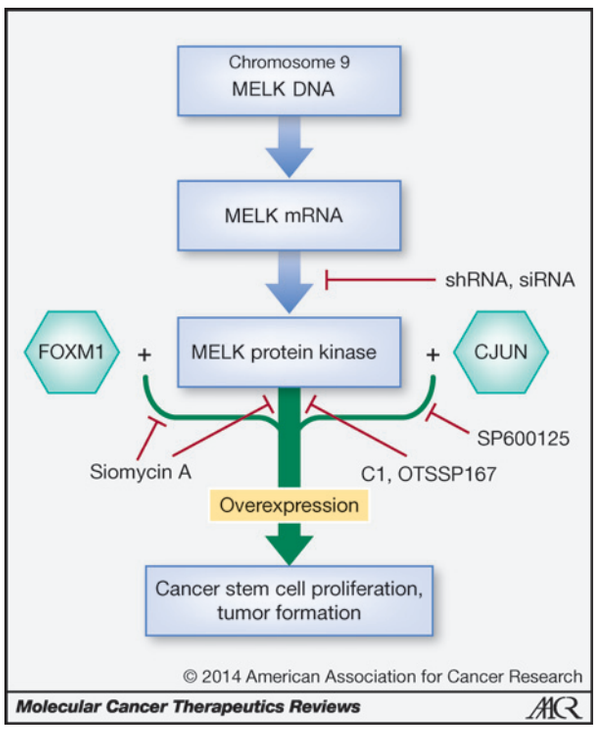

Figure 1. Schematic representation of current targets for inhibition of MELK protein synthesis and kinase activity (red markers, location of inhibition; green markers, facilitation.) shRNA and siRNA inhibit MELK mRNA from being translated into protein. Siomycin A inhibits MELK kinase activity as well as MELK–FOXM1 activity. C1 and OTSSP167 inhibit MELK kinase activity. SP600125 inhibits MELK–c-JUN activity.

SMDs and design of novel compounds represent viable strategies to pharmaceutically target MELK activity. In this context, the most recent clarification of the MELK protein structure will likely lead to further investigation of the MELK-associated signaling pathways and new mechanisms of action that may modify current approaches to targeting MELK. A summary of current and previous inhibitors of MELK and its signaling cascade are illustrated in Fig. 1. As data accumulates and interest grows surrounding MELK as a cancer culprit, current and future clinical trials of the MELK-targeting SMDs may produce valuable information about the effects and toxicities of targeting MELK for cancer inhibition, and may warrant additional investigations about MELK signaling pathways and drug development.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Grant Support

This work was supported by the American Cancer Society Grant for I. Nakano (MRSG-08-108-01), P01 CA163205-01A1, and the R01 NS083767-01. This was also supported by the American Association of Neurology Medical Student Summer Research Scholarship and by the OSUCOM Bennett Medical Student Research Scholarship for R. Ganguly.

Received September 18, 2013; revised December 31, 2013; accepted February 10, 2014; published OnlineFirst May 2, 2014.

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