

AD_____

Award Number: W81XWH-06-1-0606

TITLE: Development of Irreversible Inhibitors of MELK Kinase

PRINCIPAL INVESTIGATOR: Alexey V. Terskikh, Ph.D.

CONTRACTING ORGANIZATION: Burnham Institute for Medical Research
La Jolla, CA 92037

REPORT DATE: August 2008

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.					
1. REPORT DATE (DD-MM-YYYY) 01-08-2008		2. REPORT TYPE Final		3. DATES COVERED (From - To) 15 JUL 2006 - 14 JUL 2008	
4. TITLE AND SUBTITLE Development of Irreversible Inhibitors of MELK Kinase				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER W81XWH-06-1-0606	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Alexey V. Terskikh, Ph.D. E-Mail: terskikh@burnham.org				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Burnham Institute for Medical Research La Jolla, CA 92037				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT We have performed 3 rounds of in silico docking combined with in vitro MELK kinase activity assays. We have identified 5 independent scaffolds capable of MELK inhibition in the range of 100nm-1um. These compounds are capable to tightly bind MELK catalytic domain as measured by DCS, ruling out the protein aggregation and other non-specific inhibitory modalities. These leads are now being developed into potent anti-MELK drugs.					
15. SUBJECT TERMS Small molecule inhibitors of Maternal Leucine Zipper Kinase; Drug development; in silico docking and modeling.					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	8	19b. TELEPHONE NUMBER (include area code)

Table of Contents

	<u>Page</u>
Introduction.....	4
Body.....	4-7
Key Research Accomplishments.....	7
Reportable Outcomes.....	7
Conclusion.....	8
References.....	8
Appendices.....	N/A

Introduction

Maternal Leucine Zipper Kinase (MELK) was found to be overexpressed in undifferentiated breast cancers. In a transgenic mouse model, MELK expression labels endogenous mammary tumors, the mammary gland end buds, and proliferating neural precursors in the brain (Nakano et al., 2005; Rhodes et al., 2002). Inhibition of MELK function is deleterious for several types of tumors, including human breast cancer and experimental mammary tumors (Gray et al., 2005). Among all human kinases, MELK has a unique amino acid sequence in the catalytic loop of the kinase domain. Computer modeling of the MELK catalytic loop revealed that several unusual cysteine residues reside in the ATP-binding pocket. Previously published data support the requirement of free cysteines for MELK activity (Beullens et al., 2005). The availability of free cysteines was used successfully to create irreversible covalent inhibitors of RSK kinases (Cohen et al., 2005). Very recently, we used molecular docking to design specific covalent inhibitors of beta-catenin and non-steroidal antagonists of the androgen receptor (submitted).

We propose that irreversible covalent inhibitors of MELK kinase will efficiently destroy mammary tumors in vitro and in vivo in animal models and allow further the development of therapeutic drugs to treat breast cancer in a clinical setting.

We will identify initial compounds by in silico docking into the MELK catalytic loop, synthesize and assay candidates for inhibitory activity using surrogate and physiological targets of MELK. We will develop second/third generations of covalent MELK inhibitors (IC₅₀ 0.1-1 μ M) using iterative rounds of in silico docking and synthesis/assay of compounds and physiological targets of MELK.

Body

We used a variation of a ligand-assisted protein structure optimization protocol. Briefly, ATP molecule was docked into the original MELK model. Several ATP binding modes were selected and BPMP was applied to the protein side chains in the vicinity of the molecule, creating a set of ATP binding pocket conformations. Then, the panel of known inhibitors of kinases homologous to MELK was compiled (50 compounds) and docked into previously generated MELK conformers. The top binders were assayed in vitro, and ligands exhibiting significant MELK

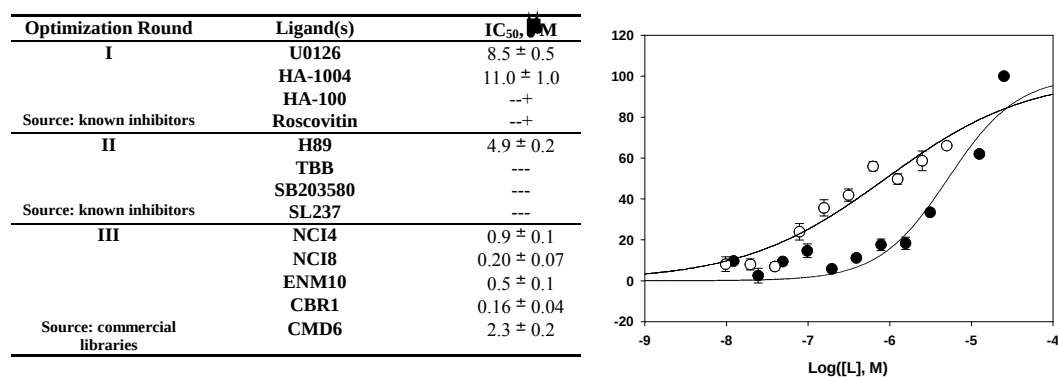


FIGURE 1. **Iterative optimization of MELK inhibitors.** **Left.** Optimization rounds. In those cases where IC₅₀ values are not available, inhibitory potency is indicated as [---] for insignificant inhibition, and [+++] for significant inhibition. For Round III, the data for only top inhibitors from a total of 50 compounds are shown. **Right.** Examples of IC₅₀ curves. Data were fit to variable slope dose response equation. 10 μ M ATP; 37°C; 1 hour incubation; 40 mM TRIS-HCl, pH 7.5; 20 mM MgCl₂; 150 mM NaCl; 0.1 mg/ml BSA; 20 μ g/ml MBP; 1 μ g/ml MELK.

inhibition (IC₅₀ < 1 mM) were selected for docking and further conformers refinement. This step in the protocol implicitly models induced fit of the protein in response to ligand binding. The protocol was applied iteratively in three rounds. The compounds in rounds I and II were selected from a panel of known kinase inhibitors, while the compound library for round III was built by searching through available commercial libraries. The search was based on Tanimoto distance from the best binders identified at rounds I and II.

The Round III compound library contained approximately 10,000 compounds. From those, 50 were purchased and screened in vitro. The examples of IC₅₀ curves for different ligands are shown on figure 1. The specificity of inhibition of certain ligands was also validated by DSC technique.

Differential scanning calorimetry (DSC) is a technique able to study thermally induced transitions, in particular, the conformational transitions of biological macromolecules. When a macromolecule changes its thermodynamic state (e.g., unfolds), a heat capacity change (ΔC_p) is observed. This change is due to the fact that the heat required to raise the temperature of a solution of unfolded protein is greater than that required for a solution of folded protein. The observed excess heat results primarily from restructuring of the solvent molecules around the side chains of the hydrophobic residues, exposed during the protein unfolding. DSC measures the

excess heat capacity of a solution (C_p) of a protein as a function of a temperature. The position

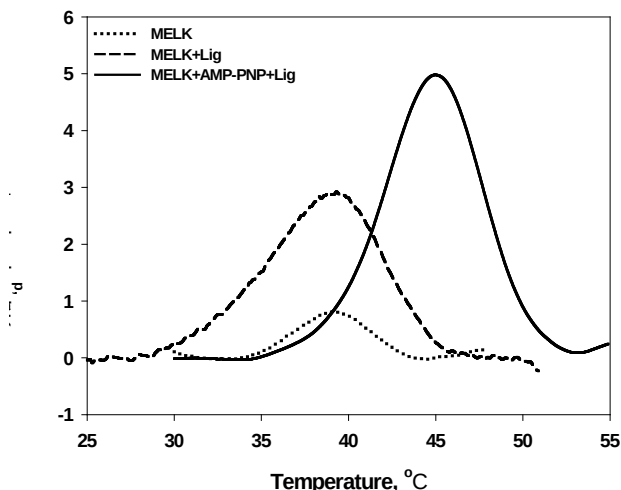


FIGURE 4. Differential scanning calorimetry experiments. MELK catalytic domain in complex with pilot inhibitor ($T_m=39.3$ °C; $\Delta H=23.0$ kcal/mol) and with both AMP-PNP and a pilot inhibitor /Lig/ ($T_m=45.3$ °C; $\Delta H=32.3$ kcal/mol).

of the transition peak corresponds to the protein melting temperature (T_m), while the area under the peak correlates with the content of ordered secondary structure of a protein. The main advantages of DSC are that it is not affected by the optical properties of the sample (as opposed to CD and fluorescence measurements), and that DSC experiments can be performed on native (unlabeled) proteins. DSC was successfully used before to characterize kinases structural state and how it affected by ligands.

Purified MELK catalytic domain was further characterized by DSC to assess the folding state of the recombinant protein (Fig. 2). The melting temperature of the MELK was found to be 38 °C with rather small area under the transition peak, which altogether suggests poor folding of the recombinant protein. However, in the presence of either non-hydrolysable ATP analog or our pilot inhibitor, significant stabilization of the protein's tertiary structure was observed, as suggested by transition peak area increase. Moreover, when both non-hydrolysable ATP analog and pilot compound were added to the protein, 6 degrees increase of melting temperature was achieved.

Task 2. Develop a second/third generation of compounds based on the initial leads using iterative rounds of in silico docking and assay/synthesis of compounds. (Months 6-10):

Key Research Accomplishments

Task 1. Identify initial set of compounds through in silico docking and assay MELK activity using surrogate and native phosphorylation targets.

In order to perform Task 1, we have: First, generated and characterized MELK catalytic domain and NusA-MELK and GST-MELK fusion proteins, critical for developing MELK inhibitors; Second: screened ~50 compound and generated 5 independent MELK inhibitors with the IC₅₀ ranging from 1 μM to 100 nM. We have did not proceed to the screening of another 50 compounds because of the virtual docking space saturation making it unnecessary continuing the primary screening. Instead, we focused our efforts on the second round of screening.

Task 2. Develop a second/third generation of compounds based on the initial leads using iterative rounds of in silico docking and assay/synthesis of compounds.

a. based on the results the refined/modified structures of active compound followed by another round of in silico docking. (Months 6-7).

b. repeat step in Task 2a to obtain a panel of diverse inhibitors with IC₅₀ around 1-10 μM

We have performed 2 rounds of screening and generated the compounds in the 2nd and 3^d rounds. We generated 4 compounds with IC₅₀ below the projected IC₅₀ (1 μM), in particular one compound with the IC₅₀ 0.16 μM, which is ~7 times better than projected.

Task 3 was not performed as planned due to the unforeseen low stability of MELK catalytic domain and thus the necessity to first characterize the binding of obtained compounds using the advanced protein stability assay (DSC).

We demonstrated that MELK inhibitors inhibit the catalytic function of MELK kinase and showed that this inhibition is due to a tight binding / protein stabilization and not protein aggregation (the DCS data).

Reportable Outcomes

We have obtained 5 MELK inhibitors, 4 of them with the IC₅₀ below 1 μM.

The list of obtained inhibitors: NCI4, NCI8, CDM6 CDR1 ENM10.

This work was the basis for an R21 application that has been currently reviewed.

The manuscript is currently being prepared. This funded supported a salary of a talented postdoctoral fellow Anton Cheltsov, PhD.

Conclusion

The completed research lays a foundation for generation of lead compounds inhibiting the MELK catalytic function. We have made a seminal discovery generating a set of unique small molecule scaffolds which will be further refined in my laboratory to generate the small molecule inhibitors of MELK catalytic function useful for research and clinical application.

References

- Beullens, M., Vancauwenbergh, S., Morrice, N., Derua, R., Ceulemans, H., Waelkens, E., and Bollen, M. (2005). Substrate specificity and activity regulation of protein kinase MELK. *J Biol Chem* 280, 40003-40011.
- Cohen, M. S., Zhang, C., Shokat, K. M., and Taunton, J. (2005). Structural bioinformatics-based design of selective, irreversible kinase inhibitors. *Science* 308, 1318-1321.
- Gray, D., Jubb, A. M., Hogue, D., Dowd, P., Kljavin, N., Yi, S., Bai, W., Frantz, G., Zhang, Z., Koeppen, H., *et al.* (2005). Maternal embryonic leucine zipper kinase/murine protein serinethreonine kinase 38 is a promising therapeutic target for multiple cancers. *Cancer Res* 65, 9751-9761.
- Nakano, I., Paucar, A. A., Bajpai, R., Dougherty, J. D., Zewail, A., Kelly, T. K., Kim, K. J., Ou, J., Groszer, M., Imura, T., *et al.* (2005). Maternal embryonic leucine zipper kinase (MELK) regulates multipotent neural progenitor proliferation. *J Cell Biol* 170, 413-427.
- Rhodes, D. R., Barrette, T. R., Rubin, M. A., Ghosh, D., and Chinnaiyan, A. M. (2002). Metaanalysis of microarrays: interstudy validation of gene expression profiles reveals pathway dysregulation in prostate cancer. *Cancer Res* 62, 4427-4433.