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Inhibition of the HEG1-KRIT1 interaction increases KLF4 and KLF2 expression in endothelial cells

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INTRODUCTION

- Cerebral Cavernous Malformations (CCMs) are a disease characterized by vascular lesions and thin-walled, abnormal blood vessels in the neurological tissue of the brain and spinal cord (1).
- These lesions result in fragile and leaky vasculature, making affected people more prone to hemorrhagic strokes, seizures, and other neurological issues (1).
- CCMs are an autosomal dominant disease that occur in about 0.5% of the general population and can be familial or sporadic (2, 3).
- The KRIT1 gene is involved in normal vascular development, and when mutated, is known to cause the formation of CCMs (3).
- The HEG1 transmembrane protein interacts with the KRIT1 protein at endothelial cell junctions, which is critical for cardiovascular development (4).
- A reduction in the interaction between HEG1 and KRIT1 increases endothelial expression of KLF4 and KLF2, which upregulates a signaling axis that confers vascular integrity and vasoprotection (5-12).
- *Krit1*$^{-/-}$ and *Heg1*$^{-/-}$ mice demonstrate severe vascular defects and lethal hemorrhage (13-15).
- In this project, a high-throughput screening assay was developed to identify compounds as potential inhibitors of the HEG1-KRIT1 interaction.
- Our findings indicate that HKi2 is a *bona fide* covalent reversible inhibitor by orthosterically competing with HEG1 for binding to the KRIT1 FERM domain.
- The HEG1-KRIT1 Inhibitor HKi2 holds promise as a new tool to study acute disruption of endothelial HEG1-KRIT1 function, and it may provide insight into early signaling events that regulate vascular homeostasis.

METHODS

- We used a flow cytometry assay to assess effectiveness of various compounds as inhibitors of the interaction between the KRIT1 and HEG1 proteins. A BD Accuri Flow Cytometer was used for this assay.
- To purify the HEG1 protein, a biotinylated peptide tag (Avi-tag) was added to a His6-Avi-tagged HEG1 peptide. The protein was then cloned, expressed in cells, and purified by nickel-affinity chromatography.
- To prepare and purify the KRIT1 protein, a His6-EGFP-KRIT1 FERM domain was similarly cloned, expressed in cells, and purified by nickel-affinity chromatography.
- SPHERO Neutravidin beads were washed and incubated with the biotin-tagged HEG1 protein for use in the flow cytometry assay.
- The EGFP-KRIT1-FERM domain was incubated with the same Neutravidin beads at room temperature for 15 minutes. The inhibitor was added to some samples, while DMSO vehicle was added to others as the negative control.
- In the presence of an inhibitor that blocks the interaction between EGFP-KRIT1 and the HEG1-bound beads, the fluorescence will not be associated with the beads, resulting in a lower signal intensity in the flow cytometer.
- Endothelial cells were treated with HKi2 and the RNA was extracted to assess changes in KLF4 and KLF2 expression levels by qPCR.

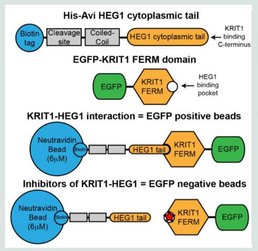

Figure 1. Illustration of the HEG1 and KRIT1 proteins bound to Neutravidin beads. The EGFP tag on the KRIT1-FERM domain allows for fluorescent signaling of the protein. In the absence of an inhibitor, the KRIT1 protein will bind with the HEG1 tail, resulting in fluorescent beads. With an inhibitor, binding is blocked, and the flow cytometer will detect less fluorescence as a result of reduced binding between KRIT1 and HEG1.

RESULTS

- A miniaturized version of the flow cytometry bead assay allowed us to screen many compounds in a 384-well format. Of 6,026 screened compounds, we identified four confirmed hits.
- HKi1 demonstrated promising activity with an IC_{50} of ~10 uM, but its limited aqueous solubility prevented us from assessing its activity at more saturated conditions (Fig. 1).
- Deconstructing HKi1 into its components led us to two substructure compounds HKi2 and HKi3, which also demonstrated inhibition of the HEG1-KRIT1 interaction at an IC_{50} of ~3.5 uM (Fig. 2).
- The small size, reduced lipophilicity, and improved solubility of HKi2 compared to HKi1 suggest that this compound can be considered as a starting point for further analysis (Fig. 2).
- The crystal structure of the KRIT1 FERM domain shows three key lysine residues in the HEG1 binding pocket. Mutating these lysine residues significantly reduced HEG1 binding to the KRIT1 FERM domain in our flow cytometry assay (Fig.3-4).
- Crystallization of the KRIT1 FERM domain in the presence of HKi1 confirmed that this compound occupies the same binding site as the HEG1 peptide and suggests that the KRIT1 lysine residues are important for HKi1 and HKi2 binding.
- In endothelial cells treated with HKi2, KLF4 and KLF2 mRNA levels were upregulated. Increasing the concentration of HKi2 resulted in dose-dependent upregulation of KLF4 and KLF2 mRNA levels (Fig. 5A-D).

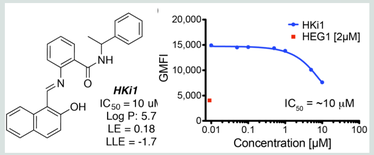

Figure 1. Structure of HKi1 and activity in bead assay. HKi1 demonstrated promising inhibitory activity of the KRIT1-HEG1 complex. The compound's limited aqueous solubility prevented us from assessing its activity at higher concentrations.

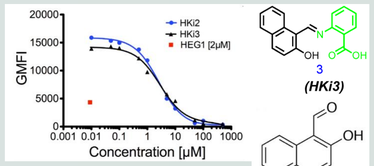

Figure 2. Structure and activity of HKi2 and HKi3. The substructure compounds HKi2 and HKi3 also demonstrated inhibitory activity at a lower IC_{50} and improved aqueous solubility in our bead assay.

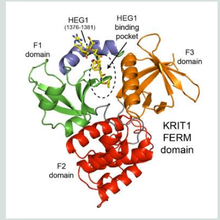

Figure 3. Structural Analysis of the HEG1 protein interacting with the KRIT1 FERM domain. The crystal structure of the KRIT1 FERM domain reveals a HEG1 binding site between the F1 and F3 subdomains (16-17).

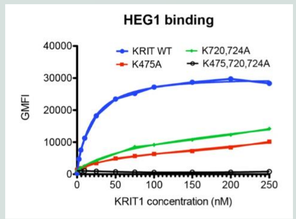

Figure 4. Specific Lysine residue mutations alter HEG1 binding. Mutations to lysine residues in the HEG1 binding pocket of the KRIT1 FERM domain significantly reduce the ability for HEG1 to bind.

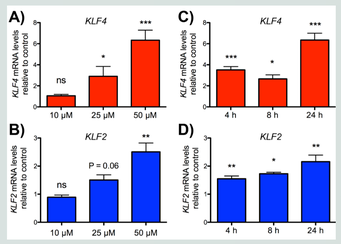

Figure 5. Effect of HKi2 on KLF4 and KLF2 mRNA levels in endothelial cells. HKi2 increased KLF4 and KLF2 mRNA levels in endothelial hCMEC/D3 cell lines. Increased concentration of HKi2 increased mRNA expression for KLF4 and KLF2 (A, B). Incubating cells with 50uM of HKi2 induced rapid upregulation of mRNA levels as early as 4 hours, and increasing up to 24 hours after treatment (C, D.)

CONCLUSION

- This project utilizes structural insights and a novel flow cytometer screening assay to identify and assess various inhibitors of the KRIT1-HEG1 protein interaction.
- These newly identified small molecule inhibitors are promising pharmacological tools that can be used to investigate the signaling pathways following the disruption of the KRIT1-HEG1 protein interaction.
- The effect of the HKi2 inhibitor in endothelial cells demonstrate that the KRIT1-HEG1 interaction is intimately linked to various transcription factors involved in endothelial response to blood flow.
- Small molecule inhibitors that can inhibit the KRIT1-HEG1 interaction are highly desirable as research tools and can be explored as future therapeutic options for vascular homeostasis and to mimic blood flow.

REFERENCES

1. Cerebral Cavernous Malformation information page. National Institute of Neurological Disorders and Stroke website. Updated March 27, 2019. Accessed January 13, 2021.
2. Cerebral Cavernous Malformation. National Institutes of Health website. Updated January 1, 2021. Accessed January 13, 2021.
3. Gunel M, Laurans MS, Shin D, et al. KRIT1, a gene mutated in cerebral cavernous malformation, encodes a microtubule-associated protein. *Proc Natl Acad Sci U S A*. 2002;99(16):10677-10682.
4. Gingras AR, Liu JJ, Ginsberg MH. Structural basis of the junctional anchorage of the cerebral cavernous malformations complex. *J Cell Biol*. 2012;199(1):39-48.
5. Cuttano, R., Rudini, N., Bravi, L., Corada, M., Giampietro, C., Papa, E., Morini, M. F., Maddaluno, L., Baeyens, N., Adams, R. H., Jain, M. K., Owens, G. K., Schwartz, M., Lampugnani, M. G., and Dejana, E. (2016) KLF4 is a key determinant in the development and progression of cerebral cavernous malformations. EMBO molecular medicine 8, 6-24
6. Zhou, Z., Rawnsley, D. R., Goddard, L. M., Pan, W., Cao, X. J., Jakus, Z., Zheng, H., Yang, J., Arthur, J. S., Whitehead, K. J., Li, D., Zhou, B., Garcia, B. A., Zheng, X., and Kahn, M. L. (2015) The cerebral cavernous malformation pathway controls cardiac development via regulation of endocardial MEKK3 signaling and KLF expression. Dev Cell 32, 168-180
7. Zhou, Z., Tang, A. T., Wong, W. Y., Bamezai, S., Goddard, L. M., Shenkar, R., Zhou, S., Yang, J., Wright, A. C., Foley, M., Arthur, J. S., Whitehead, K. J., Awad, I. A., Li, D. Y., Zheng, X., and Kahn, M. L. (2016) Corrigendum: Cerebral cavernous malformations arise from endothelial gain of MEKK3- KLF2/4 signalling. Nature 536, 488
8. Lopez-Ramirez, M. A., Fonseca, G., Zeineddine, H. A., Girard, R., Moore, T., Pham, A., Cao, Y., Shenkar, R., de Kreuk, B. J., Lagarrigue, F., Lawler, J., Glass, C. K., Awad, I. A., and Ginsberg, M. H. (2017) Thrombospondin1 (TSP1) replacement prevents cerebral cavernous malformations. J Exp Med 214, 3331-3346
9. Renz, M., Otten, C., Faurobert, E., Rudolph, F., Zhu, Y., Boulday, G., Duchene, J., Mickoleit, M., Dietrich, A. C., Ramspacher, C., Steed, E., Manet-Dupe, S., Benz, A., Hassel, D., Vermot, J., Huisken, J., Tournier-Lasserve, E., Felbor, U., Sure, U., Albiges-Rizo, C., and Abdelilah-Seyfried, S. (2015) Regulation of beta1 integrin-Klf2-mediated angiogenesis by CCM proteins. Dev Cell 32, 181-190
10. Huddleson, J. P., Ahmad, N., Srinivasan, S., and Lingrel, J. B. (2005) Induction of KLF2 by fluid shear stress requires a novel promoter element activated by a phosphatidylinositol 3-kinase-dependent chromatin-remodeling pathway. J Biol Chem 280, 23371-23379
11. Parmar, K. M., Nambudiri, V., Dai, G., Larman, H. B., Gimbrone, M. A., Jr., and Garcia-Cardena, G. (2005) Statins exert endothelial atheroprotective effects via the KLF2 transcription factor. J Biol Chem 280, 26714-26719
12. Fisher, O. S., Deng, H., Liu, D., Zhang, Y., Wei, R., Deng, Y., Zhang, F., Louvi, A., Turk, B. E., Boggon, T. J., and Su, B. (2015) Structure and vascular function of MEKK3-cerebral cavernous malformations 2 complex. Nat Commun 6, 7937
13. Whitehead, K. J., Plummer, N. W., Adams, J. A., Marchuk, D. A., and Li, D. Y. (2004) Ccm1 is required for arterial morphogenesis: implications for the etiology of human cavernous malformations. Development 131, 1437-1448
14. Kleaveland, B., Zheng, X., Liu, J. J., Blum, Y., Tung, J. J., Zou, Z., Sweeney, S. M., Chen, M., Guo, L., Lu, M. M., Zhou, D., Kitajewski, J., Affolter, M., Ginsberg, M. H., and Kahn, M. L. (2009) Regulation of cardiovascular development and integrity by the heart of glass-cerebral cavernous malformation protein pathway. Nat Med 15, 169-176
15. D'Angelo R, Alafaci C, Scimone C, et al. Sporadic cerebral cavernous malformations: report of further mutations of CCM genes in 40 Italian patients. *Biomed Res Int*. 2013;2013:459253.
16. Gingras, A. R., Puzon-McLaughlin, W., and Ginsberg, M. H. (2013) The structure of the ternary complex of Krev interaction trapped 1 (KRIT1) bound to both the Rap1 GTPase and the heart of glass (HEG1) cytoplasmic tail. J Biol Chem 288, 23639-23649
17. Gingras, A. R., Liu, J. J., and Ginsberg, M. H. (2012) Structural basis of the junctional anchorage of the cerebral cavernous malformations complex. The Journal of cell biology 199, 39-48
18. Gingras, A. R., Liu, J. J., and Ginsberg, M. H. (2012) Structural basis of the junctional anchorage of the cerebral cavernous malformations complex. J Cell Biol 199, 39-48