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TITLE: EphB1 as a Novel Drug Target to Combat Pain and Addiction

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14. ABSTRACT The synaptic receptor protein known as EphB1 is a central player in nerve injury-induced neuropathic pain and the related pain symptoms associated with the withdrawal from opioid/morphine addiction. Our hypothesis is that postsynaptic EphB1 participates in pain through the ability of its extracellular domain to form protein-protein interactions with its presynaptic ligand, ephrin-B2, and the NR1 subunit of the postsynaptic NMDA receptor to inappropriately strengthen the synapses in the spinal cord that transmit pain signals into the brain. Our project is to carry out high-throughput screens (HTS) to identify small molecular weight drug-like compounds from a >200,000 complex library that antagonize EphB1 protein-protein interactions. While we originally set out to target the EphB1:NR1 protein-protein interaction, we changed directions in Year 2 due to technical difficulties and focused on the interaction of EphB1 with ephrin-B2. In Year 3 we developed a robust HTS assay and screened the full 200,000 compound library for antagonists that disrupt the EphB1:ephrin-B2 interaction. We identified two highly related lead compounds from the 200,000 compound library and are presently focusing on characterizing them further.					
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1. **INTRODUCTION:**

Damage to pain sensing peripheral nerves following traumatic injury or other insult, such as diabetic neuropathy or bone cancer growth, strongly elevates the expression of presynaptic ephrin-B2 ligand in the nociceptive peripheral nerve fibers and postsynaptic EphB1 receptor in the dorsal horn neurons in the spinal cord. This elevated expression leads to enhanced formation of protein-protein interactions between presynaptic ephrin-B2 and postsynaptic EphB1 which leads to greater interactions between EphB1 and the NR1 subunit of the NMDA receptor in postsynaptic structures of spinal dorsal horn neurons and this drives an increase in long-term potentiation (LTP) of these synapses. The increased LTP triggers enhanced transmission of pain impulses that project into the brain, leading to classic neuropathic pain states. Similar mechanisms likely explain the severe withdrawal symptoms from the highly addictive opioid family of drugs (e.g. morphine, hydrocodone, and heroin). Whether due to nerve damage and/or withdrawal from opioid-based drugs, neuropathic pain is a serious problem faced by active military personnel, veterans of past service, as well as the general civilian population.

As described in the submitted application, our plan is to carry out high-throughput screens (HTS) to identify small molecular weight drug-like compounds from a >200,000 complex library that antagonize protein-protein interactions made with the postsynaptic receptor EphB1. While we originally set out to target the EphB1:NR1 protein-protein interaction, we changed directions in Year 2 due to technical difficulties and focused on the interaction of EphB1 with its presynaptic ligand, ephrin-B2. In Year 3 we made excellent progress and conducted a full HTS of the 200,000 compound chemical drug library available at UT Southwestern to identify antagonists that disrupt the EphB1:ephrin-B2 interaction. As described below, from the 200,000 compound library we identified 2 highly related chemicals that inhibit the EphB1:ephrin-B2 interaction and have conducted a number of additional secondary assays to gain a preliminary assessment of their effectiveness. We anticipate the discovery of these 2 related compounds will form the basis of a new class of 'smart' drugs that will be much more effective at preventing or reversing chronic pain and to help us deal with the spiking increases in addiction to opioids which has led to large increases in overdoses and deaths.

2. **KEYWORDS:**

Nerve injury pain, chronic pain, neuropathic pain, opioid addiction, ephrin-B2 ligand, EphB1 receptor, NMDA receptor NR1 subunit, presynapse, postsynapse, LTP, protein-protein interaction, AlphaScreen assay, high throughput screen (HTS), drug discovery, antagonist, small chemical entity.

3. **ACCOMPLISHMENTS:**

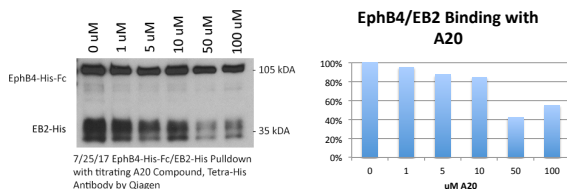
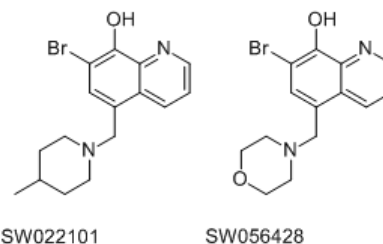
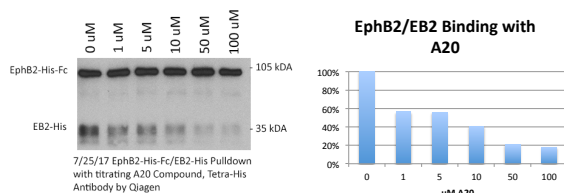
- **What are the major goals of the project?**

Because of technical difficulties expressing and purifying the protein subdomains of EphB1 (its fibronectin type 3, FN3, domains) needed to probe the EphB1:NR1 protein-protein interaction, we changed directions in Year 2 to focus attention on the protein-protein interaction of EphB1 with its partner ligand, ephrin-B2 (aka EB2). Previous studies have shown that EB2 is the presynaptic ligand expressed on the nociceptive nerve fibers that interact with the postsynaptic EphB1 receptor expressed on the dorsal horn neurons which together form the signaling complex important for EphB1 interactions

with the NR1 subunit of the NMDA receptor to drive synaptic plasticity (LTP) in the dorsal horn neurons which then signal up into the brain neuropathic pain impulses. We therefore set out to implement a HTS to probe the EphB1:EB2 protein-protein interaction and screen against a large 200,000 complex library of small drug-like chemicals for antagonists that will disrupt this interaction. Following the primary HTS, our goals are to conduct additional secondary assays to narrow down the number of initial hit compounds to a small handful of only the most potent lead chemicals and to better characterize the inhibitory activities of selected leads.

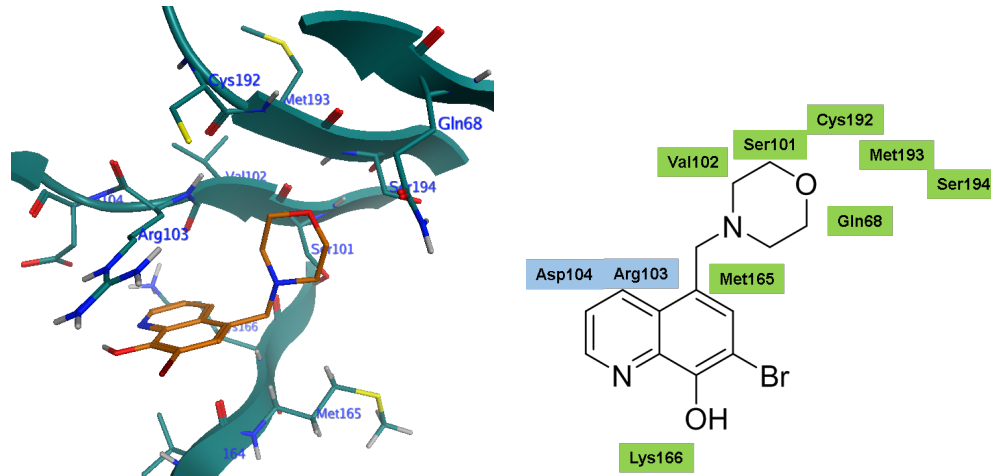
○ **What was accomplished under these goals?**

1. We conducted a full HTS screen of the ~200,000 complex chemical library using our Alpha assay and initially identified 640 possible antagonists of the EphB1:EB2 interaction.
2. We conducted confirmation assays in triplicate and a key counter screen of these 640 cherry-picked compounds and narrowed down to 32 potential lead compounds.
3. These 32 compounds were then subjected to additional counter screening assays leading to the identification of 2 highly related lead compounds, SW056428 (termed A20) and SW022010, that both appear to show strong ability to antagonize/disrupt the EphB1:EB2 interaction.
4. Focusing on compound SW056428 (A20), we have conducted additional experiments to more completely assess and characterize the activity of this small molecular weight chemical entity of as a disrupter of EphB1:EB2 interaction in our various biochemical and cell-based assays.
5. We have also conducted experiments to determine how well SW056428 (A20) might antagonize the ability of the EphB2 and EphB4 receptors to bind to EB2 ligand. Samples of the pull-down data shown below suggest SW056428 (A20) is a potent dose-dependent inhibitor of EphB1 (shown in previous quarterly report) and EphB2 but much less so towards EphB4, consistent with the fact that the neuronal EphB1 and EphB2 receptors are more related to each other than to the EphB4 receptor, which is non-neuronal and has functions in vascular endothelial cells.



6. We have also carried out computational analysis of potential docking/binding of chemical SW056428 (A20) onto the ephrin-B binding surface of the EphB2 receptor ectodomain crystal structure which we previously determined a number of years ago (Himanen et al., 1998; Himanen et al., 2001). Here, we find that SW056428 (A20) is

likely docking via Van der Waals interactions on the surface of the EphB1/EphB2 receptors made up of the amino acids that form the G strand and J-K loop, with some involvement of the D-E loop, E strand, and M strand. Importantly, these residues of EphB1/EphB2 are all important components of the EphB receptor ectodomain surface area involved in Eph:ephrin receptor:ligand dimerization and subclass specificity.



SW056428 (A20) docked into EphB2 ectodomain crystal structure. Residues within 3.5 Å of SW056428 (A20) are shown in stick representation: Gln68 (E strand), Ser101, Val102, Arg103, Asp104 (G strand), Met165, Lys166 (J-K loop), and Cys192, Met193, Ser194 (M strand). On the right schematic representation the color represents type of interaction – (Green) Van der Waals, (Blue) cationic as well as Van der Waals.

- **What opportunities for training and professional development has the project provided?**

Dr. Henkemeyer provides day-to-day, one-on-one guidance and training of all individuals working on the project, including Melody Karsi, lab manager Frances Sprouse, and Dr. Asim Bepari. UT Southwestern also provides a large and diverse faculty and seminars for advanced professional development.

- **How were the results disseminated to communities of interest?**

As we are just in the beginning stages of characterizing these novel compounds, we have not publically disseminated any of our data.

- **What do you plan to do during the next reporting period to accomplish the goals?**

We are presently in EWOFF as we have conserved funds over the past three years and have enough funds remaining to keep Melody Karsi and Frances Sprouse working on the project for another 3 months or so. They will keep working on additional biochemical and cell-based experiments to get as much data on SW056428 (A20) and SW022010 compounds in the short-term while I continue to seek out new funding opportunities to continue with project long-term.

4. **IMPACT:**
- **What was the impact on the development of the principal discipline(s) of the project?**

The major impact of our studies to date is the discovery of compounds SW056428 (A20) and SW022010 that antagonize the EphB1:ephrin-B2 protein-protein interaction. We hope SW056428 (A20) and SW022010 have great future impact by allowing us to identify new drug compounds that will modulate the presynaptic-postsynaptic interactions of these important synaptic players involved in the formation of neuropathic pain states and opioid withdrawal pain.

- **What was the impact on other disciplines?**

While our studies are still in their early stages, I believe our work will impact general fields of receptor biology and cell-cell signaling. Moreover, as we are now targeting the EphB1:ephrin-B2 receptor:ligand interaction, potential hit compounds we discover may have more wide-spread utility outside of the nervous system and could thus impact other fields where these molecules have functions, such as in vascular system development and remodeling (e.g. tumor angiogenesis).

What was the impact on technology transfer?

While our studies are still in their early stages, we certainly hope we are able to identify strong antagonists of the EphB1:ephrin-B2 protein-protein interaction. If we are indeed successful, there will be great potential to impact technology transfer as we would have our hands on a new class of drug-like compounds that could be further developed into actual new drugs to treat and/or prevent chronic pain conditions caused by nerve injury and/or withdrawal from opioid abuse.

- **What was the impact on society beyond science and technology?**

As stated above, our high-risk research has the potential to product a high-impact result, new drugs to combat pain and addiction.

5. **CHANGES/PROBLEMS:**

- **Changes in approach and reasons for change**

No problems or changes to report.

- **Actual or anticipated problems or delays and actions or plans to resolve them**

The only really anticipated problem is a lack of future funding for the project to keep moving forward. Drug discovery is a very labor intensive, time consuming, and costly endeavor and we are grateful for the past 3 years of funding from the USAMRMC for this important work. It is extremely difficult to obtain funds for such high-risk early phase research on drug discovery. And while the competition for the small amount of grants available is intense, though I hope that with our success at identifying unique

chemical compounds SW056428 (A20) and SW022010 we will be able to convincingly demonstrate that we are on the right path.

- **Changes that had a significant impact on expenditures**

None.

- **Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents**

None.

- **Significant changes in use or care of human subjects**

N/A

- **Significant changes in use or care of vertebrate animals.**

N/A

- **Significant changes in use of biohazards and/or select agents**

N/A

6. **PRODUCTS:**

- **Publications, conference papers, and presentations**

Nothing to report.

- **Website(s) or other Internet site(s)**

Nothing to report.

- **Technologies or techniques**

See above.

- **Inventions, patent applications, and/or licenses**

We have been in discussions with the UT Southwestern Development office concerning IP on SW056428 (A20) and SW022010. There is interest, though no patent applications have been submitted at this time.

- **Other Products**

Nothing to report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS:

- **What individuals have worked on the project?**

Name:	Mark Henkemeyer
Project Role:	PI
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	6
Contribution to Project:	Worked on all aspects of project.
Funding Support:	
Name:	Frances Sprouse
Project Role:	Technician
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	6
Contribution to Project:	Lab manager, assisted on all aspects of project.
Funding Support:	
Name:	Melody Karsi
Project Role:	Technician
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	12
Contribution to Project:	Expressed and purified proteins from bacterial cells, conducted biochemical protein-protein interactions assays experiments on purified proteins, develop Alpha assays, conducted HTS screens, conducted secondary screens, conducted biochemical protein-protein interactions assays, and worked on cell-based experiments.
Funding Support:	
Name:	Asim Bepari
Project Role:	Postdoctoral fellow
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	9
Contribution to Project:	Expressed and purified proteins from mammalian cells, conducted biochemical protein-protein interactions assays, and worked on cell-based experiments to investigate ephrin-B:EphB1:NR1 protein-protein interactions

	in mammalian cells.
Funding Support:	

- **Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**

Nothing to report.

- **What other organizations were involved as partners?**

Nothing to report.

8. SPECIAL REPORTING REQUIREMENTS:

COLLABORATIVE AWARDS:

N/A

- **QUAD CHART:**

See attached

9. APPENDICES:

N/A

EphB1 as a Novel Drug Target to Combat Pain and Addiction

1115013

Clinical & Rehabilitative Medicine



PI: Dr. Mark Henkemeyer

Org: University of Texas, Southwestern Medical Center

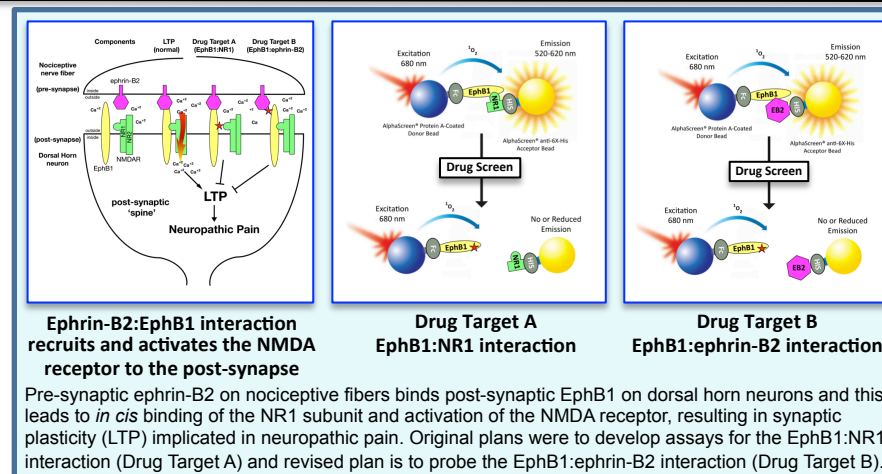
Award Amount: \$1,385,682

Study/Product Aim(s)

- Aim 1 (revised): To screen a library of small drug-like compounds to identify those that antagonize the EphB1:ephrin-B2 protein-protein interaction.
- Aim 2 (revised): To synthesize and evaluate EphB1-binding peptoids for ability to antagonize the EphB1:ephrin-B2 protein-protein interaction.

Approach

A sensitive AlphaScreen chemiluminescent assay for use on 384 well plates was developed to probe the EphB1:ephrin-B2 interaction in high-throughput. This assay was used to screen a library of >200,000 small drug-like chemicals, resulting in the identification of lead compounds that disrupt the interaction. We have also characterized the antagonistic activities of peptoid compounds converted from known peptide inhibitors of EphB.



Accomplishments: We have screened a 200,000 complex library for small molecular weight drug-like compounds that inhibit the EphB1:ephrin-B2 protein-protein interaction and have identified 2 highly related lead chemical compounds. We are presently focusing much effort on further characterizing the antagonistic properties of these two compounds and other related analogs.

Timeline and Cost

Activities	CY	14	15	16	17
Develop Alpha/Peptoid screens		█			
Execute Alpha/Peptoid screens			█		
Validate antagonistic compounds				█	
Estimated Budget (\$K)		\$435K	\$434K	\$517K	\$000

Updated: (UTSW, September 25, 2017)

Goals/Milestones(revised)

CY14-15 Goals – Develop protein reagents and interaction assays

- Clone vectors to make EphB1/FN3 and NR1/NTD domains
- Purify large amounts of EphB1 and NR1 proteins from bacteria
- Establish EphB1-NR1 AlphaScreen assay and other assays

CY15-16 Goals – Develop interaction assays and initiate HTS screens

- Optimize protein-protein HTS interaction assays
- Begin HTS screens

CY16-17 Goals –HTS and validation

- Complete HTS of 200,000 complex library
- Conduct biochemical and cell-based tests of promising lead hits
- Comments/Challenges/Issues/Concerns**

- We have conserved funds as we worked to overcome earlier challenges and have made great progress.

Budget Expenditure to Date

Projected Expenditure: \$129,197 (Y3Q4) /\$1,385,682 (Y1Q1-Y3Q4)

Actual Expenditure: \$115,686 (Y3Q4) /\$1,362,364 (Y1Q1-Y3Q4)