Membrane Proteins: Critical Issues in Therapeutic Development

Introduction: Membrane proteins as a class represent a key target for pharmaceutical intervention. G-protein coupled receptors (GPCRs), ion channels and viral receptor proteins represent obvious examples, with GPCRs alone representing the target of ~1/3 of developed drugs. As a class, membrane proteins remain challenging to approach with greater hurdles than water soluble proteins and often remain poorly characterized relative to their physiological and therapeutic importance.[1] New, more sophisticated, approaches to analyze their structures have helped to better understand and develop therapeutics that target them.[1] However, their research and development costs sometimes can be unpredictable and outsized due to the difficulty in processing appropriate grade material for analysis.

Challenges: Cells as a rule have low endogenous levels of most key membrane proteins, thus making isolation or even isotope labeling difficult. In turn, this limits their isolation for sophisticated techniques like x-ray crystallography or isotopic labeling for NMR. Sometimes non-native expression systems are used to bridge this gap, even though they may create some uncertainty regarding the proper folding of the structure, or proteins may lack key structural features like proper glycosylations.[1] Second, due to the membrane component, protein purification or even isolation of an enriched membrane fraction are challenging, requiring sophisticated knowledge, skills and abilities (KSAs).[1] Third, many common assays for receptors like GPCRs often rely on readout on association between a tagged molecule to a tagged receptor expressed on the cell surface, most commonly a cell-based FRET assay where the receptor and small molecule both have their respective fluorescent tags. While mutated and tagged structures are important tools in protein studies, controls are needed for such mutations and additions to proteins that can affect biological function. A functional assay that used native (unmodified) protein structures that could replace assays like FRET might prove more useful for many drug development studies.

Proposed Solution: The easy availability of usable quantities of pure membrane protein resolves one of the more time-taxing issues for structural determination projects. A recent series of publications demonstrated this advantage with the rapid characterization 5-HT_3 receptor.[2–7] In line with platform development described below, the protein was expressed and purified.[7] Purified 5-HT_3 was used to create a single chain llama antibody (nanobody) against the receptor and this nanobody was then used to stabilize 5-HT_3 for crystallization to perform x-ray crystallography.[2] Electron tomography was also used to further elucidate the structure and conformational changes of this receptor.[3,5,6] In many cases, nanobodies helped stabilize crystal structures for analysis; however, they also have potential as therapeutics.

eEnzyme now provides off-the-shelf stable cell lines expressing membrane proteins of particular interest to the research and pharmaceutical communities as a platform. Upon induction conditions, cell lines express their respective membrane protein to very high levels. Their high expression can easily serve in isotope labeling for NMR, or as a source for purifying the larger amounts needed for techniques like x-ray crystallography. Presently there

are about 250 human membrane proteins in the library, as well as some representatives from other species such as rat and mouse.

Alternatively, the platform can readily provide material as a membrane fraction or purified protein, according to preference. KSAs for the unique challenges of working with membrane proteins was transferred to a large number of individual species during development. That means re-development time of re-acquiring these KSAs is eliminated for researchers acquiring membrane fractions or purified protein from this platform. In particular, this avoids lengthy startup for projects. Proteins are expressed with tags to simplify the isolations if in-house purification processes from cell lines are preferred instead. TEV protease cleavage sites are present to allow complete removal of the tags after purification.

There are a number of other useful features as well. Both CHO and HEK-293 parental strains are available. The availability of the human HEK-293 as parental cell line for human membrane protein expression means it will be properly folded with proper post-translation modifications when those issues are a consideration. Additionally, eEnzyme can provide access to experts for custom projects including x-ray structure determination of the membrane protein in question or nanobody development. Other custom projects include development for a presently unlisted protein, which can still take advantage of KSAs generated from the platform setup.

In addition, eEnzyme compliments these platforms with another platform for an off-the-shelf cell-based functional assay for GPCRs, the ACTOne assay. Using fluorescent reporter based system that detects changes in cAMP, these assays follow this biological shift in HEK-293 cells created from the signal transduction of the respective native GPCRs. This functional assay can serve as a control in comparison to the mutated form, supplement such data, or be used as the preferred screening platform in its entirety over a FRET cell based assay for simultaneous assessment of biological output and specificity in early drug target screening. The assay is high-throughput compatible for this purpose, and uses equipment that is normally available in most laboratories that do such work, further controlling costs and development time. Assays are compatible for small molecule drug evaluation and discovery, or antibody targeting of GPCRs. Alternatively, drug screening with ACTOne can be outsourced to eEnzyme if preferred.

Technical Specifications:

Cells expressing membrane protein have high level expression (generally over 1 million per cell). They are tetracycline inducible and well suited for ^{13}C, ^{15}N labeling. Removable tags allow for purification by streptactin and/or nickel/cobalt column.

For all membrane preparations for which radio ligand binding characterization has been conducted, we deliver 1µg/unit, as soon as 1µg is enough to obtain 1000 specific DPM at the Kd concentration of the radio ligand of reference. For all membrane preparations for which radio ligand binding characterization has not yet been conducted, we deliver 10µg/unit.

Membrane receptors are available in purified form, and x-ray structure discovery is available as a custom project.

For ACTOne GPCR assays, HEK-293-CNG cells express a modified Cyclic Nucleotide Gated (CNG) channel that opens in response to elevated intracellular cAMP levels and consequently result in ion flux and cell membrane depolarization. A fluorescent dye is used to detect this depolarization.

Target Market: In a proteome atlas of the 672 developed drug targets, 250 of these are integral membrane proteins and 28 have both secreted and membrane forms {https://www.proteinatlas.org/humanproteome/tissue/druggable}. Also, membrane proteins are often the receptors for viral entry, and proper targeting with antibodies or small molecules can block viral entry, and such molecules are potential new anti-viral therapeutics, as for example with influenza [8]. Enhanced tools for membrane proteins will lead to improved drugs for known targets and important new leads at reduced costs. They will also help physical and biological characterization for basic research needs.

Summary: This document outlines two platforms that can reduce costs and speed up projects for research and development for structural, biological and therapeutic studies of membrane proteins. The first platform provides membrane proteins in off-the-shelf formats, greatly decreasing costs and development time. The second platform allows the functional assay of native GPCRs. Additionally, custom services are available for to carry this through to structure discovery or GPCR assay projects. These better tools will help leverage other developments taking place for membrane proteins, such as new structural tools.[1]

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