Cryo-EM accelerates drug discovery by delivering previously intractable structural insights

Cryoelectron microscopy (cryo-EM), a powerful biophysical technique that can analyse molecular assemblies, is changing the paradigm in structure-based drug design. As targets become more challenging, solving structures of drug target molecules or biologics becomes increasingly difficult. Cryo-EM empowers drug discovery teams to take advantage of rational drug design for many more major drug target classes, opening a pathway to best-in-class drugs.

Cryo-EM allows the structural analysis of protein complexes flash-frozen in their near native states. One can now directly visualise not just large macromolecules, but also smaller proteins complexes, including membrane proteins. This powerful technique can be used to complement traditional methods, such as X-ray crystallography (XRD) or nuclear magnetic resonance (NMR) for structure-based drug discovery.

Cryo-EM enables structural analysis of protein complexes, providing better insight into all classes of biomolecules, including proteins that are difficult to work with. This method reveals detailed structural features of targets, ligands and their interactions at the atomic level, reducing the development time and guesswork of lead compound design.

Additionally, by capturing a series of intermediate states from a reaction mixture, ‘time-resolved’ EM is possible, providing structural information over the course of a reaction.

Conformational analysis of these intermediates provides unique, physiologically-relevant details of disease mechanisms. Even intractable targets are laid bare by cryo-EM. You can now analyse sample amounts that are generally too small for other techniques. No crystallisation, concentrated solutions or epitope labelling are needed, meaning ion channels, transporters and receptors can now be analysed. Also, membrane protein complexes in their native lipid environment can now be observed, thanks to lipid nanodiscs combined with cryo-EM single particle analysis (SPA). Atomic models can be obtained with cryo-EM due to the combination of ultra-stable microscopes with automatic cryogenic sample handling, cameras that are fast and super sensitive, high-performance image processing procedures and modern computational power. The acquisition of such models enables faster lead discovery and better lead optimisation.

This article is intended to introduce you to the
versatility of cryo-EM and to the variety of benefits made possible with this method. Based on the results of these applications, you can determine if the adoption of cryo-EM for your projects can lead to an increase in the number of valid results, thereby improving your rational design efforts.

A substantial number of pharmaceutical companies have recently published papers providing proof of how cryo-EM delivered critical structural insights. In several cases, cryo-EM provided the insight that was unattainable with other methods, thus accelerating the path to drug discovery.

In this article we describe the benefits of cryo-EM in accelerating drug discovery and feature pharma use cases that recently have been published.

Applications of cryo-EM in Pharma

Protein drug target classes

A drug target is a molecule in the body, usually a protein, that is intrinsically associated with a particular disease process and that could be addressed by a drug to produce a desired therapeutic effect (from Comprehensive Medicinal Chemistry II, 2007). Common classes of drug targets are proteins and nucleic acids. The definition can refer to the biological target of a pharmacologically-active drug compound, the receptor target of a hormone (such as insulin), or some other target of an external stimulus. In this article we present examples of how cryo-EM reveals the structure of biological drug targets which are most commonly proteins such as membrane proteins (GPCRs), ion channels, enzymes and hormone receptors. Cryo-EM was also crucial to reveal the structure of the virus binding sites and the influence of small molecules to a large protein drug target assemblies.

GPCRs

G protein-coupled receptors (GPCRs) are membrane proteins that regulate a variety of cellular responses to external stimuli. They are involved in a wide range of diseases and are a critical drug target; more than 50% of modern protein-based pharma is related to GPCRs. As membrane proteins, they are large, flexible and resistant to crystallisation, meaning traditional analytical techniques such as NMR and XRD cannot be used to characterise their structures. A collaborative project performed in conjunction with Dr Mazdak Radjainia, a staff scientist at Thermo Fisher Scientific, successfully employed cryo-EM to analyse the GPCR structure. In addition, researchers were able to capture the dynamic nature of these complexes, as structural families can be grouped and individually characterised using cryo-EM. Class B GPCRs are major targets for treatment of chronic disease, including diabetes and obesity. Structures of active receptors revealed that peptide agonists engage deep within

![Figure 1](image.png)

Cryo-EM density map of a GPCR complex: glucagon-like peptide-1 receptor (GLP1R) is an important target for treatment of Type 2 diabetes. Most GLP1R agonists are injectable drugs and therapeutic potential of this receptor is far from exhausted. Using cryo-EM it was possible to identify a new agonist pocket for non-peptides. This result will help to find new oral medicines that will be safer and better in protecting from Type 2 diabetes.
the receptor core allowing G protein interaction and activation. The solved structure of a novel non-peptide agonist bound to the GLP-1 receptor identified an unpredicted non-peptide agonist binding pocket (Figure 1).1

Ion channels
Ion channels, as the primary means of transport and exchange between cells, are a key subject of research. They critically impact the function of many diseases; however, they are challenging therapeutic targets because they are very difficult to crystallise. Once extracted from their native membrane environment, ion channels’ structures collapse.

Cryo-EM can reveal not only the structure of an ion channel in its native lipid environment but also the various discreet open and closed states, which are critical to understanding ion gating mechanisms.

“Cryo-EM is undoubtedly the technique of choice to use for determining multiple conformation states of ion channels by imaging the proteins in different conditions,” said Carus Lau, postdoctoral scientist at the Victor Chang Cardiac Research Institute.2

Cryo-EM complements crystallography in unravelling selective sodium channel blockers

“Using cryo-EM, we are tackling many targets that otherwise would be impossible to visualise. Nav1.7, for example, is only one of these cases.” (Quote from the SelectScience editorial by Dr Claudio Ciferri, Senior Scientist and Head of Cryo-EM at Genentech.)

Voltage-gated sodium (Nav) channels are targets of disease mutations, toxins and therapeutic drugs. Mutations in Nav channel subtypes are associated with migraines, epilepsy, pain and cardiac and muscle paralysis syndromes. Channel blockers lack subtype selectivity and have not been well understood.

In this research the objective was to determine key structural templates to design selective Nav channel antagonists using spider protoxin-II (ProTx2).3

Cryo-EM analysis independently validated the crystallographic structural model of the receptor

Figure 2
Cryo-EM structures clearly show structural differences of ProTx2 bound to activated (blue, magenta) versus deactivated (black) receptor. Image based on PDB-entry 6n4r and 6n4q created with PyMol by Hans Raaijmakers

Figure 3
Leishmania tarentolae proteasome 20S subunit. Alpha type subunits in yellow, Beta type subunits in green, endopeptidase in orange, GSK3494245 in blue. Images based on PDB-entry 6qm7 created with PyMol by Hans Raaijmakers
site. While the crystallographic structure took many years to solve and soaking in compounds was not successful, the cryo-EM was the ultimate method of choice. In addition, cryo-EM structure supported mechanistic interpretation of ProTx2 complex shifting the activation of Nav and pharmacologically stabilising the closed-channel state (Figure 2).

Enzymes
Cryo-EM structures reveal new, potent, selective inhibitor of parasite enzyme
Visceral Leishmaniasis (VL) or Black Fever is one of the most severe tropical diseases with up to 40,000 deaths annually. Present treatments have serious drawbacks of prolonged treatment duration and low tolerability, with sometimes high teratogenic effects. Also, the significant geographical variations in effectiveness have been noticed. The additional serious drawbacks are high costs of treatment and strict conditions for cold storage.

A new GSK preclinical candidate inhibits chymotrypsin-like activity over the human enzyme and selectively inhibits the parasite enzyme.

The objective of this research was to identify a low-cost, safe, effective, oral, short-course drug for VL based on inhibition of parasite enzyme.

High-resolution cryo-EM structures revealed previously undiscovered binding site for inhibitors of chymotrypsin-like activity. (Figure 3).4

Cryo-EM reveals allosteric site for enhanced ‘drugability’ of ATP-citrate Lyase
ATP citrate lyase (ACLY) is a cytosolic enzyme that catalyses conversion of mitochondrially-derived citrate into acetyl-CoA and oxaloacetate, thereby defining the first step in the cellular fatty acid syn-
thesis pathway. ACLY is an interesting target for anti-cancer drugs because cancer cells depend on its activity for proliferation. It is also the target against dyslipidaemia and hepatic steatosis. In this research by Nimbus Therapeutics, the structure-activity relationship (SAR) of targets is explained through binding modes of ACLY tetramers.

The objective was to study previously undiscovered allosteric ACLY binding site as target for enhanced ‘druggability’ of inhibitors.

This was the first ever high-resolution ACLY structure where cryo-EM, along with computational insights, determined full tetrameric ACLY structure with small molecule ‘druggable’ inhibitor (Figure 4).

Hormone receptors
Insulin binding
Medical advances have made diabetes a manageable disease, providing its sufferers with longer, healthier lives. However, until recently, the binding mode of insulin to its receptor was unknown. Knowing the precise mechanism of binding could lead to the development of more effective therapeutics.

“The insulin receptor (IR) is a dimeric protein that plays a crucial role in controlling glucose homeostasis, regulating lipid, protein and carbohydrate metabolism, and modulating brain neurotransmitter levels. IR dysfunction has been associated with many diseases, including diabetes, cancer and Alzheimer’s Disease,” said Dr Giovanna Scapin, Senior Principal Scientist, Merck & Co.

Dr Scapin has spent years studying insulin and diabetes. She was also one of the first adopters of cryo-EM. Thanks to single particle analysis, her

Figure 6: Cryo-EM structure of BK polyomavirus-like particle in complex with single chain antibody. Images of PDB-entry 6GGP were created with PyMol by Hans Raaijmakers

Figure 7: Cryo-EM structure of the stalled RNC showing Ribosome, 40S light blue, 60S grey mRNA in red, tRNA in green and blue, nascent chain in magenta with the inhibitor PF846 in blue. Image of PDB-entry 6ole created with PyMol by Hans Raaijmakers
team was able to ascertain the precise interaction between insulin and the insulin receptor, opening up exciting new avenues in drug discovery (Figure 5).

**Viruses and antibodies**

Cryo-EM reveals structure of viral epitope, enabling development of new antibody screen against Polyomaviruses

Human polyomaviruses (BK and JC) establish persistent infection in kidneys during childhood with minimal clinical manifestations. The virus reacts under immunodeficient conditions, causing nephropathy and haemorrhagic cysts.

Novartis developed a new high-throughput, functional antibody screen to examine the response to polyomavirus with isolated monoclonal antibodies that neutralise BK and JC virus subtypes.

The objective of this research was to identify a complex binding site of the virus capsid protein, which was not possible with crystallography due to the complex's quaternary structure.

Cryo-EM reveals the quaternary nature of viral epitope and unravels potent modality for inhibiting polyomavirus infection in kidney transplant recipients and other immunocompromised patients (Figure 6).

**Small molecules**

How cryo-EM illuminates stalling ribosome by small molecules and opens new therapeutic strategy?

One of the new strategies for therapeutic development is using small molecules that inhibit protein synthesis by selectively stalling the ribosome. Structures of human Ribosome Nascent Chain (RNC) complexes, stalled by drug-like molecule PF846, selectively blocks the translation of a small number of proteins by an unknown mechanism.

The objective in this research by Pfizer was to determine the structures of RNC complexes which could not be solved by X-ray crystallography due to their size, complexity and conformational flexibility, establishing structural foundation for developing therapeutic small molecules inhibitors.

“The requirement of X-ray crystallography is that you need to obtain a diffracting crystal to get atomic resolution. That has been a big hurdle if you’re working on challenging targets. Almost every target we’re working on now is very challenging,” said Dr Seungil Han, Associate Research Fellow at Pfizer.

High-resolution cryo-EM structures reveal how small molecule PF846 selectively stalls the translation of the human ribosome by binding in the ribosome exit tunnel and altering the path of the nascent polypeptide chain.

“The cryo-EM method is well-suited for providing critical structural information to influence the discovery of biologics, vaccines and gene therapies. However, the most surprising aspect has been our ability to provide binding site and binding pose information for small molecules on large proteins or protein complexes,” said Dr Han (Figure 7).

Cryo-EM-enabled development of new small-molecule inhibitor for therapy against Paroxysmal Nocturnal Hemoglobinuria (PNH)

Paroxysmal Nocturnal Hemoglobinuria (PNH) is a rare, acquired and life-threatening disease leading...
to the premature death of all blood cells. Human complement component 5 protein (C5) is a validated drug target in an already available anti-C5 antibody approved therapy.

The objective of research, conducted by Novartis, was to first obtain the small-molecule inhibitor of C5 complement protein as the new more potent therapy with identified and optimised mechanism of action.

Growing crystals suitable for X-ray crystallography was unsuccessful and cryo-EM had to be used in experiments to narrow in on the binding region of the protein (Figure 8)9

Hans Raaijmakers has been a structural biologist since 1993. He started his career as a structural biologist (EMBL, Heidelberg, Universidade Nova in Lisboa, University of Utrecht). He then spent 14 years working in the fields of crystallography, drug design and modelling in pharma for Organon, Schering-Plough, MSD and Lead Pharma. Hans now specialises in the development of MicroED at ThermoFisher.

Dr Mazdak Radjainia is an expert in pharma cryo-EM with 15 years of experience in academia and industry. His present responsibilities in Thermo Fisher Scientific Pharma EM team include leading various strategic partnerships aimed at pharma customer enablement by streamlining gene-2-structure workflows, particularly for difficult membrane proteins. His particular interest is in developing transferable GPCR cryo-EM workflows as part of GPCR cryo-EM and drug design collaboration with Monash University. Further objectives cover increasing throughput, ease-of-use and amenability of small proteins for cryo-EM.

Dr Hervé-William Remigy completing his PhD in Biophysics at the Biozentrum at the University of Basel in 2001 focusing on the structural analysis of membrane protein complexes using Electron Microscopy (EM). He then worked on EM automation and developed Cryo-EM sample preparation by flash freezing. Since 2009 he has worked at Thermo Fisher Scientific and currently supports Pharma customers as a Business Development Manager.

Aleksandar Stefanovic has a scientific background in chemical crystallography. He is experienced in the development of pharma market sub-segments and new business models, with more than 25 years of extensive work in corporate operations, sales, marketing and business development in the analytical pharma sector. Aleksandar’s present responsibility in Thermo Fisher Scientific is pharma market development and organisational growth in Europe, North America and the Asia Pacific region.

References