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Meeting Report

Experimental and computational methods for enabling structure-based drug discovery. Highlights from The Society for Medicines Research Symposium

Cambridgeshire, UK – March 14, 2019

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Summary

This 1-day meeting of the U.K. Society for Medicines Research focused on multidisciplinary approaches for using three-dimensional structures of proteins in drug discovery programs. Most protein structures are determined using one or more of the following techniques: X-ray crystallography, NMR and cryo-electron microscopy. In addition, computational predictions of protein structures and analysis of protein–ligand and protein–protein interactions can also help guide many stages of structure-based drug research, from sample preparation, to structure determination and drug design. This meeting explored the interplay between experimental and computational methods for driving drug development programs that make use of molecular protein structures. Presentations, drawn from academia and industry, described state-of-the-art and emerging experimental methods used by structural biologists. Complementary computational approaches were also presented that not only facilitate structure determination, but also harness the vast information available on protein structure, function and ligand interactions to accelerate drug discovery processes. A recurring topic was the need for developing improved methods for structure determination of membrane proteins. Although challenging to handle experimentally, these proteins offer numerous opportunities for creating new drugs for many medical indications including cancer, cardiovascular disease, neurological conditions and pain.

Key words: Crystallography – Cryo-electron microscopy – NMR – Free electron laser (FEL) – Database – Biophysics – Membrane protein – GPCR

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Protein–Ligand Complex Binding Pocket Structure Determination with *N*MR², NMR Molecular Replacement Approach

Julian Orts, Department of Chemistry and Applied Biosciences, ETH Zürich, Switzerland

Julien Orts opened the Society for Medicines Research (SMR) meeting with a talk on an elegant method for determining structural information of ligands bound to protein. This NMR-based method has the benefit of not needing

crystals but requires timescales more akin to those expected of crystallography. A total of two-thirds of the 134,000 X-ray structures in the PDB have been solved by molecular replacement, in which we make use of homologous structure to solve the crystallographic phase problem. In comparison, there are only 12,000 structures solved by NMR, as there is no equivalently "easy" way to solve a structure by NMR. In NMR, one must start with labeled protein, which is not always straightforward to produce. The subsequent protein assignment process can take weeks to months, and it can only be done for limited protein sizes. To solve an NMR structure in the time and with the ease of crystallography, the protein assignment process should be skipped, which is where *N*MR^2 arises. The principle of *N*MR^2 is shown in Figure 1 (1, 2). In drug discovery, the ligand binding site is generally the interesting part of the protein–ligand complex, so the method focuses on characterizing this site and thus circumnavigates the long and tedious protein assignment step. As for the X-ray molecular replacement, the *N*MR^2 requires a model of the protein (apo structure, homologous structure, etc.) in addition to the ligand of interest, and the only experimental data collected are the NOE-derived distances from standard NMR experiment, interrogating the methyl groups within the binding site and their proximity to the bound ligand. This is a 100% restraints-driven structure calculation where *N*MR^2 permutates its way through the signals to arrive at a model which explains all the data. As the signal comes from the protonation of methyl groups, it can be used for higher molecular weight proteins (from G protein-coupled receptors [GPCRs] to mega Dalton proteins) which are normally considered out of reach of NMR structure determination.

New Computational Methods and Online Resources for GPCR Drug Discovery

David Gloriam, Department of Drug Design and Pharmacology, University of Copenhagen, Denmark

GPCRs form one of the largest protein families in the human genome. They modulate nearly all biological processes and are the targets of 34% of approved drugs. The number of structures and associated data for these proteins is increasing rapidly and is now captured within the GPCRdb.org (Fig. 2), including drug interactions and indications, mutations, 3D structures/models and constructs (3).

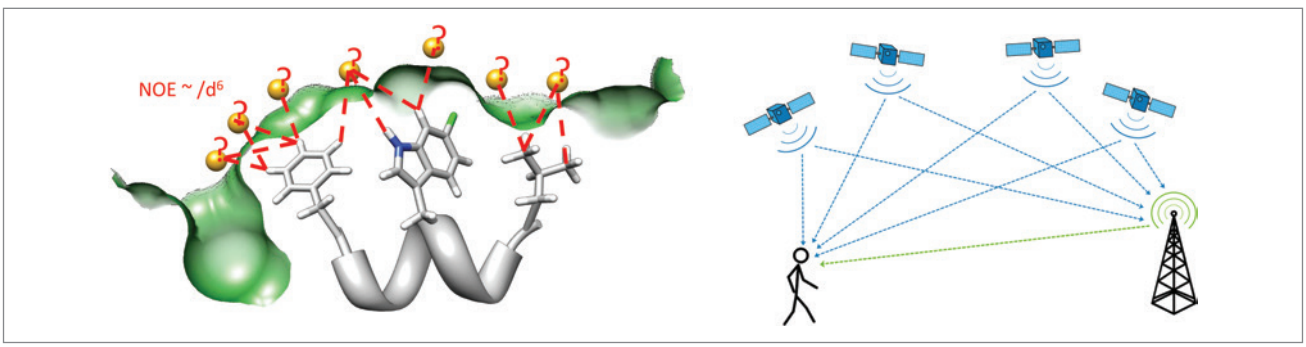

Figure 1. The principle of *N*MR^2. Nuclear Magnetic Resonance Molecular Replacement (*N*MR^2) derives the complex structure of the binding site within a few days without protein resonance assignment and using only standard NMR experiments. *N*MR^2 localizes the ligand binding site and the ligand orientation like a GPS would do. The experimental input data are ligand intra- and ligand–protein inter-molecular distances derived from NOEs (red dashed lines) between the unassigned protein protons (usually methyl groups) and the ligand protons. (Figure derived from images supplied by Dr. Julien Orts, ETH, Zürich, Switzerland.)

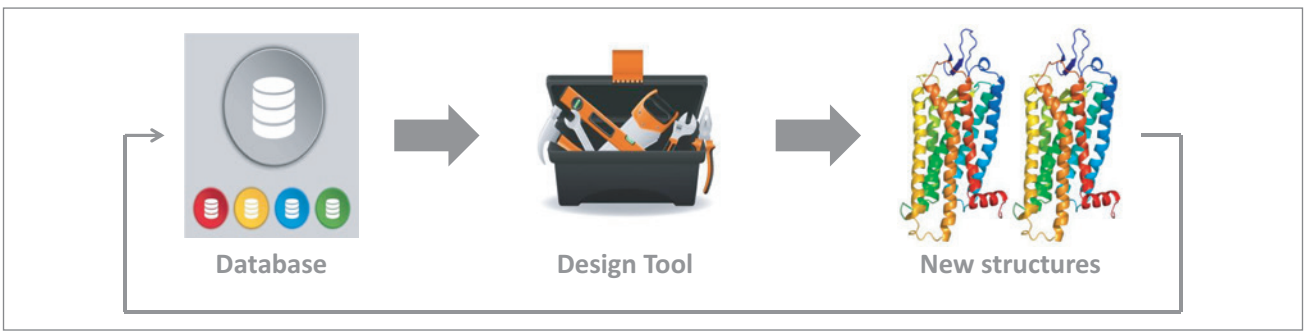

Figure 2. The GPCRdb online resource. (GPCRdb, http://www.gpcrdb.org) integrates information about all available G protein-coupled receptor (GPCR) structures in the Protein Data Bank along with information about mutations and other biochemical studies relevant to GPCR structure determination. The result is a collection of design tools that can be used to improve experimental strategies for obtaining 3D structures of GPCRs using crystallographic or cryo-electron microscopy techniques. (Figure supplied by Professor David Gloriam, University of Copenhagen, Denmark.)

The 321 structures for 62 distinct receptors are provided in a browser, where you can select the most appropriate templates based on annotation of inactive/active states, complexed signal proteins and the fusion proteins used. Only 16% of GPCRs have a structure, and only 35% have a close template that can be used to build an accurate model. Structures of the active state conformation of GPCRs are still in the minority, as its flexibility makes it hard to crystallize. Several types of protein engineering are required to generate crystals. In contrast, if the structure is solved by cryo-electron microscopy (cryo-EM) this generally requires less engineering. GPCR structure determination is often done in the presence of a G protein and hence the resulting structure is in the active state.

There are common strategies for generating GPCR proteins that are amenable to crystallization. One can use information from existing GPCR structures to create strategies for obtaining the structure of GPCR (when no structure exists). Protein fusion sites, and N- and C-terminal truncations are also transferrable between these proteins. The GPCRdb presents all of this information in a single site, and allows rational construct design, with an online interactive tool where the user can generate a list of constructs with all the options, download the sequence and order the DNA constructs. The majority (59%) of stabilizing mutations in GPCRs are mutations to alanine. In cases where one of these mutations increases the stability of a GPCR, the alanine is usually replacing a small residue. The main rationale for this stabilizing effect is improved helix packing, the activation of a micro-switch that converts the protein from one state to another (active ↔ inactive), or a change in sodium ion binding that results in greater stabilization of an active state. One of the added values of the database is the annotation of all the methods and reagents from PDFs of GPCR publications, so one can see the cell line trends, the impact of expression systems, the effect of moving from DDM to LMNG detergent, etc. in one central place.

The combination of all this data is a powerful tool that allows the prediction of GPCR–ligand interactions for those whose structure have not yet been solved, including off-target prediction. The GPCRdb is an incredible resource for all GPCR knowledge and is presented to the user via an intuitive and attractive interface.

The NavMs Voltage-gated Sodium Channel as a Target for Antiepileptic, Analgesic and Antiarrhythmic State-specific Drug Design

Bonnie Ann Wallace, Institute of Structural and Molecular Biology, Birkbeck College, London, UK

Human voltage-gated sodium channels (Navs) are targets for drug design, with relevance in epilepsy, cardiac conditions and pain. To design effective and safe drugs against these targets, we need to understand the specificity and selectivity of these ion channels, which is made more complicated by the fact that compounds can bind to the open or closed state of the channel. Therefore, is it important to understand the different conformations of the protein and to be able to stabilize the appropriate conformations to allow the design of these drugs. The differences between different conformations can be fairly small. Therefore, it can be challenging to distinguish between different conformations using techniques such as circular dichroism spectroscopy as there is no change in secondary structure but rather a modest movement in the arrangement of helices.

Eukaryotic sodium channels have four helices forming the voltage sensor and two helices forming the pore. This motif of six helices is repeated four times as a single polypeptide chain. In contrast, prokaryotes have a single copy of this motif and an additional C-terminal helix. Four copies of this peptide assemble. As a result, the prokaryote and eukaryote Nav secondary structures are essentially identical. Overall there is 30% amino acid similarity between the channels. But the sequence of key residues is conserved, so the prokaryotic protein binds eukaryotic channel blockers with similar kinetics and affinities. Through years of work it has been possible to optimize crystals of the Nav channel from the prokaryote *Modestobacter marinus*, NavMs. Crystal structures of this channel have been solved in the open and activated form, in which the ion binding sites can be determined, revealing the difference between the ion binding sites in sodium (relative wide, with side chain interactions [4]) and potassium (narrow, with interactions from the backbone atoms) channels (Fig. 3) (5).

These channels have also been studied by cryo-EM, using CD again to identify stabilizing conditions which are favorable for grid generation (6). While the relatively small size of the prokaryotic protein may have been an advantage for crystallization, it makes the EM analysis more of a challenge, with the additional complication of both open and closed forms of the protein being identified from the class averages analyzed. Despite the challenges of working on these challenging proteins, it has been possible to image ligands bound to NavMs using crystallography, with site directed mutants cross validating the interactions observed between the protein and ligand. And this work points toward the opportunity to design drugs targeted toward the open or closed form of channels (7).

Crystals or Grids? Integrating Cryo-EM into the SGC Membrane Protein Pipeline

Katharina Duerr, Membrane Protein Structure + Function Group, Structural Genomics Consortium, University of Oxford, UK

The Structural Genomics Consortium (SGC) is based at six sites around the world and specializes in high-throughput structural biology applied to protein families across

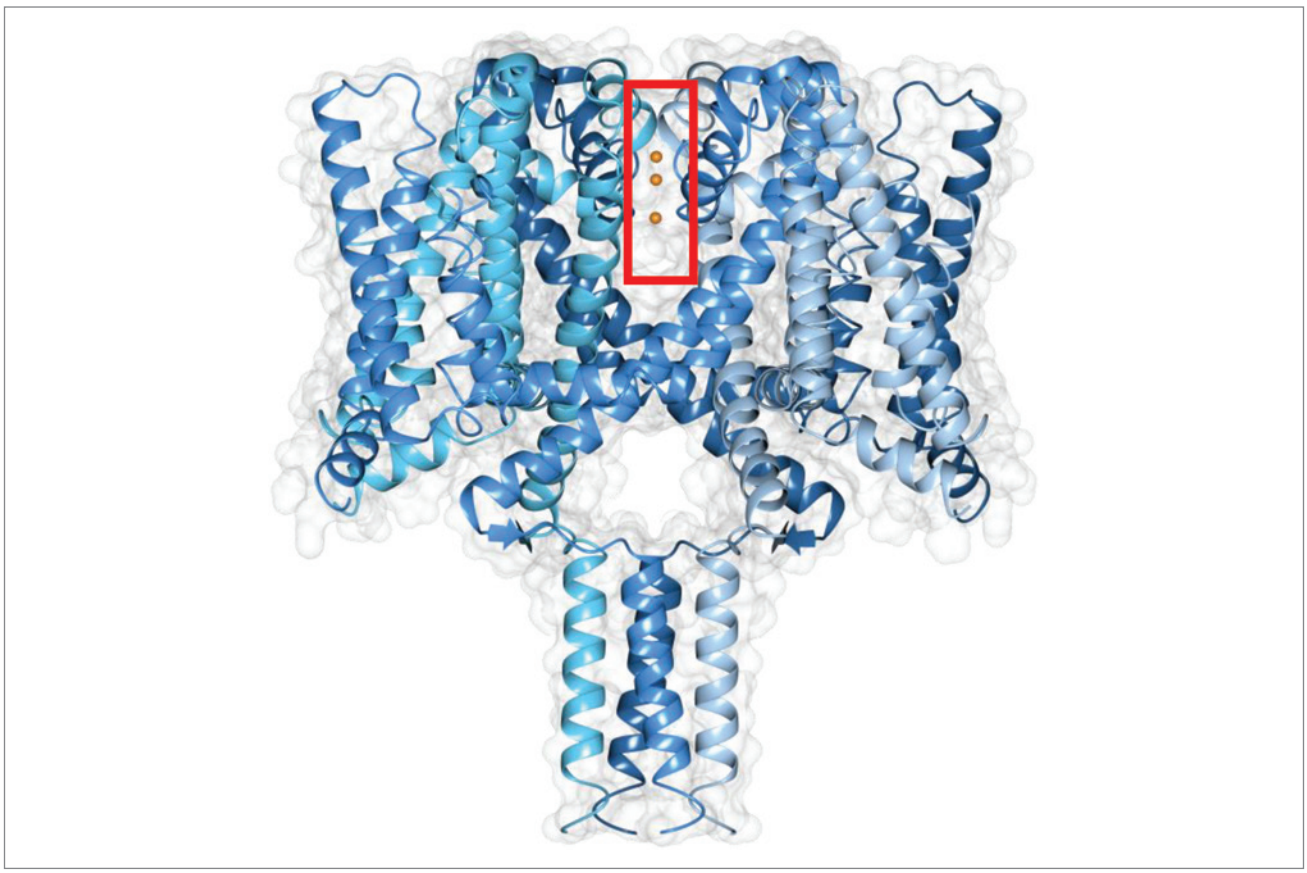

Figure 3. Crystal structure of the open, activated NavMs voltage-gated sodium channel. The three sodium ions in the selectivity filter are denoted by the orange balls within the orange box. Each of the four monomers in the functional tetrameric unit is depicted in ribbon representation in a different shade of blue, overlaid on the space filling model in grey. (Figure supplied by Professor Bonnie Wallace, Birkbeck College, London, UK.)

therapeutic areas of high interest to the drug discovery community. More than 2,000 human structures have been deposited since its inception in 2004. The SGC has also developed multiple chemical probes and tools to help validate and explore the proteins they work on.

Katharina Duerr is one of three group leaders engaged in the recombinant production and structure determination of integral membrane proteins at the SGC in Oxford, U.K. In this talk she focused on an example of a solute carrier target, SLC14A1, and an ion channel family target, TRPML1.

The solute carrier (SLC) superfamily is the largest family of membrane-bound transporter proteins in humans, with 430 members. They constitute an important family of drug targets. Some 20% of the members are associated with a disease phenotype. These proteins enable the transport of a wide range of substrates (e.g., vitamins, sugars, nucleotides, metabolites and metals) across biological membranes. To date, there have been only a handful of human SLCs structures solved to date.

The urea channel SLC14A1 (UTB-1) is a key player for urinary concentration in the kidney and prevents intracellular urea intoxication which has been implicated in several disease states (8). For example, UTB-null mice suffer from depression-like behavior due to accumulation of high levels of urea in the hippocampus formed as a byproduct of polyamine synthesis (9). These mice also show a malignant phenotype in bladder urothelium (10). Urea levels and UTB-1 expression levels are elevated in brain tissue of Huntington's disease patients (11).

The urea channel has recently been targeted as a novel diuretic mechanism based on observations that SLC14A1 knockout mice have urine concentrating defects. Compounds targeting this mechanism, known as urearetics, have potentially with fewer side effects than conventional loop diuretics (12). This approach to drug development has provided some tool compounds that were used to characterize interactions with purified SLC14A1 protein, and aid in the crystallization and structure determination of this solute carrier. The apo structure was solved to 2.4 Å resolution, but in the presence of a UTB-1 inhibitor, it was found that the putative inhibitor binding site was occupied by a detergent molecule. Current work is examining the SLC14A1 construct in detergent-free systems such as

amphipols and nanodiscs to elucidate binding interactions with inhibitors by cryo-EM.

A multi-million Euro IMI-funded initiative, ReSOLUTE, has recently been approved to accelerate the generation of solute carrier reagents and assays. This workstream has the ultimate aim of an increased understanding of signaling and regulation of solute carriers and the generation of multiple high-affinity binders (MAbs, camelid and synthetic nanobodies) as tools to facilitate further research across the family (https://re-solute.eu/).

In a different, but related, project the Duerr group have targeted lysosomal ion channel targets (13). Loss of function mutations in the mucolipin 1 channel (TRPML1) are linked to the lysosomal storage disorder mucolipidosis type IV and have been linked to neurodegenerative diseases such as Alzheimer's disease. At the outset, the project benefited from potent small-molecule ligands provided by academic and pharma collaborators. Cryo-EM structures of TRPML1 and TRPML3 in complex with small-molecule agonists were solved at 4.5 and 3.1 Å resolution, respectively. Work is continuing to solve further co-structures with a new set of agonists and antagonists to understand the molecular basis for their increased potency and selectivity.

Disease States of FGFR Kinases Probed Using NMR and Biophysics

Alex Breeze, Astbury Centre, University of Leeds, UK

Fibroblast growth factor receptors (FGFRs) are frequently upregulated or mutated in cancers and developmental disorders, making them important and compelling targets for pharmaceutical intervention (14). There are four members of the family, which collectively are high-affinity receptors for fibroblast growth factors (FGFs). FGF signaling regulates cellular proliferation, migration and differentiation, while aberrant FGF signaling can promote tumor development.

Alex Breeze is Professor of Biomolecular NMR at the Astbury Biostructure Laboratory in Leeds, UK. In his talk he described his work in Leeds and his previous work with AstraZeneca at Alderley Park, UK, on the FGFRs.

Through the extensive research carried out on the family, it was noted that FGFR kinases appear highly resistant to adopting a Type-II DFG-out conformation. Only one inhibitor, ponatinib (Fig. 4), has been described as binding to a DFG-out conformer. Ponatinib displays very slow binding kinetics with FGFR-1 as measured by surface plasmon resonance, implying a conformational selection binding event and an intrinsically impaired DFG flip rate (15). Isothermal calorimetry analysis revealed that the much more common Type-I inhibitors of the protein (e.g., PD-173074; Fig. 4) resulted in exothermic ΔH values, while Van't Hoff plots confirmed that Type-II inhibitors showed endothermic ΔH values. A later compound, tivozanib (Fig. 4), was also found to be a Type-II inhibitor and showed the same behavior in these assays.

Ponatinib was known to bind to a different kinase, Abl, some 20-fold faster than it does to FGFR-1 but with a similar off-rate (16). A closer examination of the co-crystal structures of ponatinib in Abl and FGFR-1 indicates that the binding mode and interactions with both proteins is almost

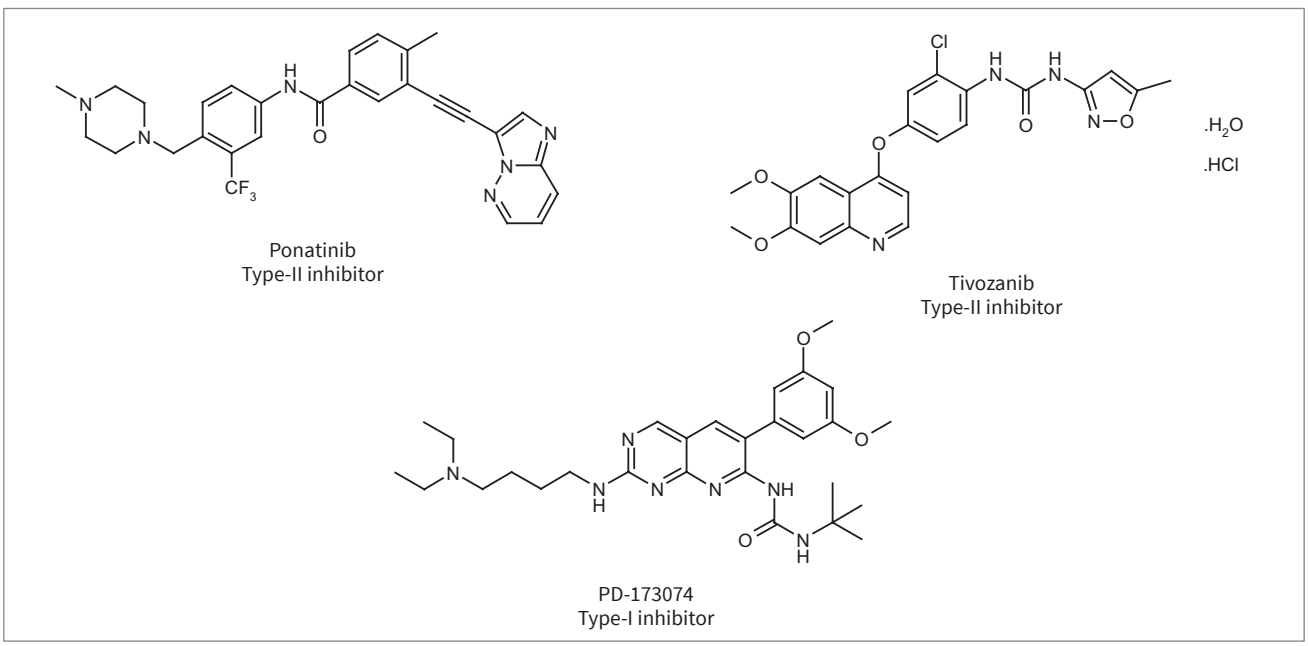

Figure 4. Fibroblast growth factor receptor (FGFR) kinase inhibitors. A key difference between inhibitors in the Type-I and Type-II forms are conformational changes associated near a structural motif containing the sequence DFG. Type-I inhibitors maintain the conformation of this region in a "DFG-in" position. Binding of Type-II inhibitors results in conformational changes that flip this motif into a "DFG-out" position.

identical but a Gln replaces an Asn in the αC-β4 loop of Abl. The location of this change is important, as it resides in the hairpin that connects the αC-β4 segments and appears to autoinhibit flipping of the DFG triad. This observation led to a hypothesis that this single residue change acts as a molecular brake toward DFG flips. Further corroborating evidence was that partially unfolded FGFR-1 protein, and protonated FGFR-1 at low pH, substantially accelerated the k_{on} for ponatinib but not for an exemplar Type-I inhibitor. DFG-in and DFG-out complexes show very distinct NMR chemical shift perturbations and different solvent exposures relative to apo protein by HDX-MS analysis. NMR analysis has allowed an elucidation of a network of allosteric control within the kinase which is now being modeled with metadynamics simulations.

Mutations in the FGFR-3 protein that are associated with disease are strong clients for the Cdc37:HSP90 chaperone system. It is notable that FGFR-3 is a much stronger client for this system than any of the other FGFR subtypes. Using complexes reconstituted from pure protein components and using SEC and pull downs for analysis, researchers observed that mutant FGFR-3 variants that are less thermostable than wild-type protein are stronger clients for the HSP90 system, for example the E466K, I538F and N540K mutations (17). Using TROSY NMR, it was shown that Cdc37 binds with mM affinity to wild-type FGFR-3. The spectrum revealed several new, sharp peaks at random-coil shifts that were assigned to the N-terminal region of the N-lobe. Comparing spectra for FGFR-3 mutations, the mutant proteins were stronger clients with much reduced K_d values and showed enhanced exchange in the N-lobe but decreased exchange in the C-lobe region upon Cdc37 binding. The most extensive C-lobe stabilization occurred for the most labile mutant (E466K). It was then shown that Cdc37 binding to the FGFR protein variants inhibited kinase activity proportionally to the client strength with the disease-associated mutated FGFR-3 proteins providing the least extent of autophosphorylation in the presence of Cdc37. A structural model based on data from small-angle X-ray scattering was then built of the interaction of Cdc37 and FGFR-3 which suggested strongly that the FGFR-3 kinase domain interacts with the Cdc37 N- and C-terminal regions. Cdc37 therefore helps to present a remodeled kinase region in FGFR-3 to HSP90 for stabilization and refolding.

X-ray Free Electron Laser: Opportunities for Drug Discovery

Michael Hennig, leadXpro, Villigen, CH

leadXpro is a biotechnology company founded in 2015. It provides a gateway for utilizing large Swiss academic technology facilities in the Paul Scherrer Institute (including the Swiss Light Source, SLS, Villigen) and the University of Basel (C-CINA, cryo-EM). It performs contract research drug discovery for the pharmaceutical industry, using a multifaceted approach that combines X-ray crystallography and cryo-EM with other biophysical and computational methods to deliver clinical lead compounds. Included within its portfolio are methods for the production of high-quality preparations of membrane proteins using its established expertise in expression and purification of proteins (18), as well as premier access to the SLS including its X-ray free electron laser (SwissFEL). The first protein structure determined at the SwissFEL facility was determined by leadXpro [(19); discussed in more detail below].

Michael Hennig is the CEO of leadXpro in Villegen, Switzerland, and also professor in structural biology at the University of Basel. In addition to providing an introduction about leadXpro, he also described how X-ray free electron lasers (XFELs) are being used for the determination of protein crystal structures and as emerging platforms for drug discovery (20).

XFELs are among the newest large-scale facilities that have been built at five sites throughout the world: the LCLS in the United States, SACLA in Japan, SwissFEL in Switzerland, PAL-XFEL in South Korea, and the European XFEL in Germany (20). There is also a new XFEL being constructed in Shanghai, China. Compared to X-ray synchrotron sources, which is most widely used by industry and academia for protein structure determination, XFELs have enormously increased peak brilliance and focusing properties. Data collection on the SwissFEL requires the preparation of microcrystals (Fig. 5A) that are injected into the system at an optimized density and flow rate (Fig. 5B) using a so-called lipidic cubic phase jet system (LCP jet; Fig. 5C). Single images of individual crystals are collected in a process known as serial crystallography (21), and data are processed to ultimately yield the three-dimensional structure of a protein (Fig. 5D). The high brilliance of the XFEL source combined with elegant sample handing and data collection facilities enable very high-quality structure determinations of proteins to be conducted at room temperatures (more physiologically meaningful compared to frozen samples) with no radiation damage (19).

As an emerging technique for drug discovery, XFEL-based structure determination is particularly advantageous for obtaining 3D structural information from challenging biological systems such as membrane proteins (20). Membrane proteins are estimated to bind 60% of marketed pharmaceutical drugs (22), though there are considerable gaps in knowledge about their 3D structures and associated drug-binding modes. Often these gaps arise because these proteins only form microcrystals that are not amenable to structure solution using traditional, synchrotron-based sources. Serial crystallography (described above) provides a “second chance” as microcrystals are capable of yielding high-quality data because of the high peak

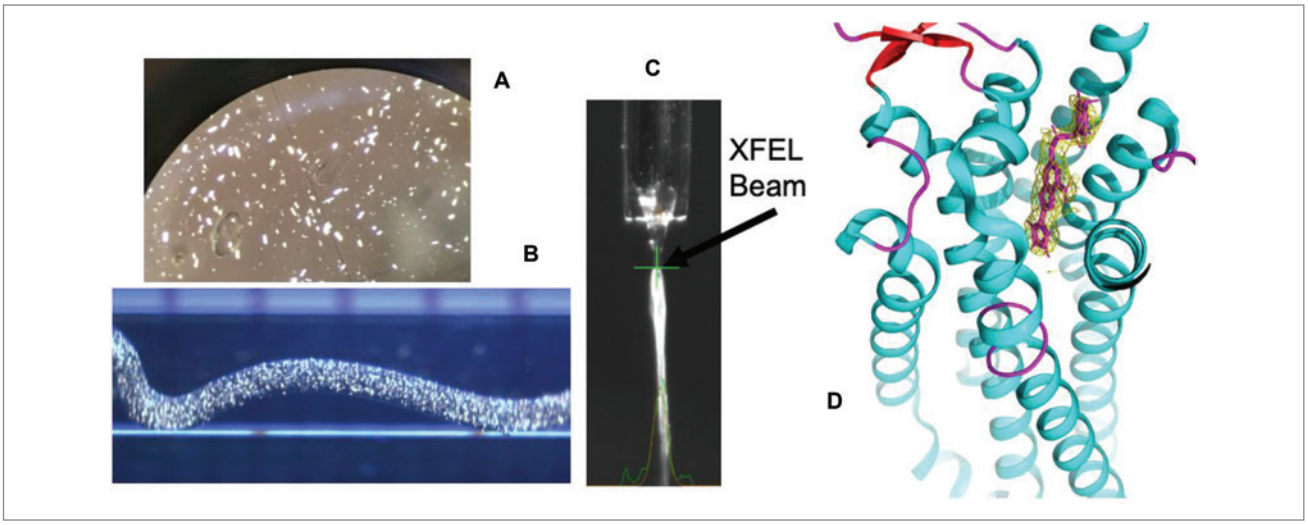

Figure 5. First biostructure experiment at the Swiss X-ray Free Electron Laser (SwissFEL). A carefully prepared sample of G protein-coupled receptor (GPCR) crystals (**A**) was delivered by an LCP jet (**B**) to the high brilliance X-ray beam (**C**) and the structure determined at 2.5 Å resolution (**D**). (Figure supplied by Professor Michael Henning, leadXpro, Villigen, Switzerland.)

brilliance and beam focus of XFEL sources. This approach thus opens up the possibility of determining the structures of a larger number of membrane proteins and membrane protein–ligand complexes. In addition, the time-resolved capabilities of XFELs also mean that more information about ligand-binding dynamics and associated conformational changes in these proteins may be obtained—all of which can contribute new insights into the drug design strategies.

Structures of Human $GABA_A$ Receptors

Radu Aricescu, MRC Laboratory of Molecular Biology, Cambridge, UK

$GABA_A$ receptors are membrane-embedded ligand-gated channels that bind the neurotransmitter γ-aminobutyric acid (GABA) as well as to a wide variety of compounds and drugs with anticonvulsant, antianxiety, analgesic, sedative and anesthetic properties (23). Many of these compounds are known to act through allosteric mechanisms, binding at sites remote from the receptor binding site but causing conformational changes that alter the functional activity of these receptors. Structural information about how these drugs bind and potentially alter the structures of most $GABA_A$ receptors is unknown. However, the emergence of cryo-EM techniques has now made it possible to obtain meaningful information about the structures of these and other membrane proteins (in complexes with compounds). This information about the structure can be used to provide new insights about physiological function and drug targeting.

Radu Aricescu is a group leader at the MRC Laboratory of Molecular Biology in Cambridge, UK. His research uses a variety of structural biology techniques, X-ray crystallography, cryo-EM and tomography, to understand how neurotransmission occurs in synapses on a molecular scale. His work includes structure determination of neurotransmitter receptors. His presentation was a genuine *tour de force* that primarily focused on how his group's studies of the $GABA_A$ receptor progressed from the first crystal structure published in 2014 (24) to recently published cryo-EM structures of the receptor in complexes with a variety of ligands that provide information about physiological function of this protein (23, 25).

Radu's group was able to obtain high-resolution structures of the intact $GABA_A$ receptor because, crucially, his group developed a reconstituted lipid bilayer environment that appears to aid in maintaining structural integrity (Fig. 6). The resulting hetero-pentameric structures did not show evidence of collapsed architectures (previously observed in some cryo-EM reconstructions). In addition, the conditions identified for cryo-EM studies also permitted comparative studies to be undertaken with a variety of different compounds that bound to the $GABA_A$ receptor. The compounds used included the channel-blocker picrotoxin, the competitive antagonist bicuculline, GABA – an agonist, and the well-known benzodiazepines alprazolam and diazepam. The cryo-EM structures of these receptor–compound complexes provided insights into the binding modes of these compounds. In addition, comparison of structures identified compound-induced structural changes, including allosteric interactions and rotational movements, that convert the receptor between "resting" and "desensitized" states. The availability of such detailed information about structure-function relationships using this "structural pharmacology" approach also creates possibilities for developing rationally designed drugs. Ideally, this type of approach to drug design can lead to new compounds with a targeted,

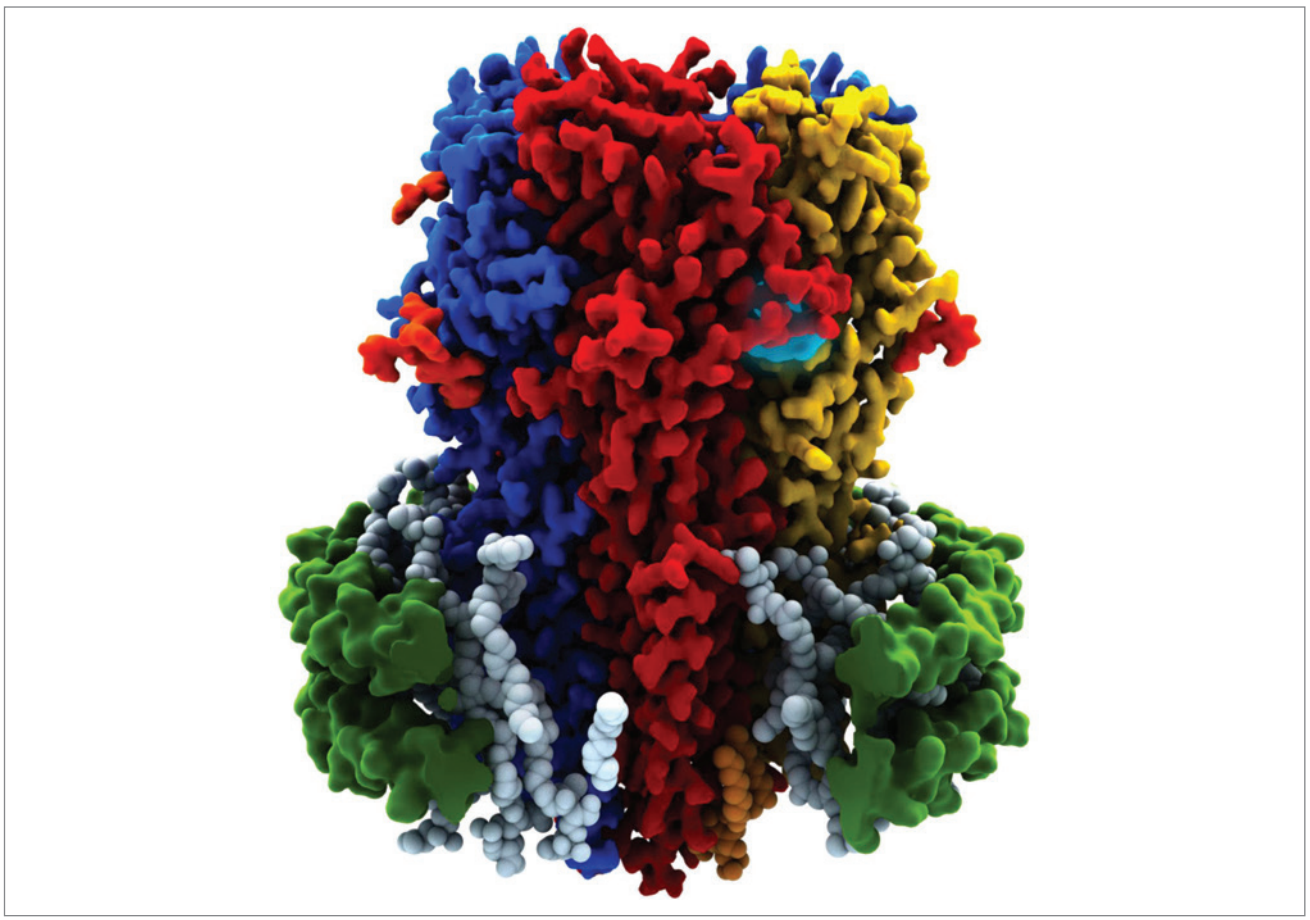

Figure 6. Human $GABA_A$ receptor structure in a lipid bilayer. The benzodiazepine alprazolam (Xanax) is shown in cyan, N-linked glycans in orange, lipids in grey, PIP2 in wheat, the nanodisc belt protein in green, and the receptor α/β/γ subunits in red/blue/yellow, respectively. (Figure supplied by Dr. Radi Aricescu, MRC Laboratory of Molecular Biology, Cambridge, UK.)

specific physiological effects and safety profiles with minimal side effects and reduced propensities for abuse.

Disclosures

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