Development of Pharmaceuticals Using Non-Crystalline Methods at Diamond Light Source

Macromolecular crystallography (MX) has been essential for the development of many pharmaceutical compounds in clinical use today. Advances in MX, such as fragment screening and serial crystallography, have led to the discovery of new drugs and the advancement of pharmaceutical pipelines. Aside from providing detailed information about molecular interactions, the MX field has contributed to large protein databases that can be used for rational drug design as well as artificial intelligence approaches for discovering new potential drug candidates.

As the name suggests, crystallography requires proteins to be in a crystalline form in order to acquire structural information. With a good quality crystal, extremely high-resolution 3D structural information can be collected on precise molecular interactions that can be used to identify potential drug targets. This has been the cornerstone of drug discovery for decades, but focusing too heavily on MX for all stages of the pharmaceutical pipeline can lead to problems. Not all drug target interactions identified via MX progress all the way through to the clinic. The chances of successfully identifying a true potential drug target are significantly increased when used in combination with a variety of different techniques that can be carried out at a synchrotron. Complementary techniques are often those that allow the user to analyze protein drug interactions in solution, in a non-crystalline form. This is due to the fact that protein molecule drug targets and drug ligands behave differently in solution than they do when tightly coordinated in a crystal lattice obtained under not identical conditions. Structural features of proteins can mean they can be more flexible in certain regions, which can dramatically affect ligand binding affinities as well as producing novel steric effects that would not be seen in a crystalline sample. Moreover, drug candidates often exist in racemic mixtures, which can also have dramatic effects on the target-ligand interactions. These factors are not accounted for in MX, which is why additional validation is required to have more certainty for how a particular drug candidate will truly interact with the target protein in a clinical situation.

Two techniques that are useful for studying protein target interactions in solution are circular dichroism (CD) and X-ray absorption spectroscopy (XAS). CD and XAS complement MX studies and provide essential validation tools for pharmaceutical development. One of the main benefits relates to sample preparation and that both CD and XAS allow for measurements to be taken in solution. This means that for drug interactions, it is possible to mimic the in-vivo environment more closely while directly observing how various drug candidates interact with their targets.

The flexibility in sample preparation is also extremely valuable when considering the formulation of drugs for clinical use. In MX, all of the sample preparation is designed to deliver high-quality crystals, without which there can be no image. This can often lead to sacrificing viable formulations for those that will contribute to crystal formation. Using complementary techniques like CD and XAS means that different formulations of drug candidates and different mixtures of proteins can be tested to better understand the effect of changing conditions on the drug interactions. It is much easier to test a variety of factors such as concentrations, shear effects, humidity, and temperature, all of which can impact the interactions of drug candidates with their potential targets.

CD and XAS also give a much more detailed view of the dynamics of interactions between drug targets and ligands. With the ability to measure non-crystalline proteins in solution, it is possible to gauge how proteins and ligands will interact under natural conditions. CD can measure the shape profile of different molecules and molecular clusters or aggregates within a solution. While relatively low resolution, it is possible to monitor the shape profiles in terms of structure elements, such as helical, twisted or flat ribbons, turns and irregular as the native fingerprint of the molecule over time, to get a dynamic view of how the whole solution changes. This can be very useful in detecting any protein misfolding and what could be done to ameliorate it or prevent it. It can give information on all of the chemical species in a mixture in one experiment. XAS is different in that this spectroscopy technique only allows for the detection of metal species. This means that if a drug or a protein doesn’t contain a metal, then it will be completely invisible. This is important in the measurement of many dynamic interactions because it allows the analysis to focus on very specific changes to the state and coordination of specific metal ions.

Techniques such as CD have some additional benefits along the pharmaceutical production pipeline. For example, for a drug to be viable, it is not only important to know how it behaves with a target protein under optimal conditions, but it is also important to know how it behaves in transport and storage. CD studies have been used to assess the viability and stability of drugs over time, temperature, and light
irradiation to ensure that they are fit for their intended purpose. If not, precautions can be taken to extend the life of the drug, either by changing the formulation or modifying the storage conditions. The utility of CD for quality control and monitoring the changes in complex mixtures over time has led to it being an important tool for quality control for the production of some compounds.

For successful outcomes of drug candidates at clinical trials, it is important to use a variety of complementary techniques to fully understand how ligands interact with their targets and other cellular components. There are many techniques that can be used, especially at a synchrotron. MX has previously been mentioned, but many diffraction-based techniques can be used, especially when trying to understand the final formulations of drug compounds ready to be used in the clinic. However, this article will focus on non-crystalline methods, as they provide an important and valuable complement to MX. They provide information that is simply not possible to obtain when proteins are immobilized and unable to perform the regular functions. Here, we will provide examples of how the non-crystalline techniques CD and XAS have contributed to pharmaceutical development at different clinical stages. The article will also discuss how machine upgrades will affect the potential of these techniques in the future and the new ways in which they could contribute to pharmaceutical development.

Circular dichroism

Beamline B23 (Figure 1) at Diamond Light Source (Figure 2) uses UV light and the circular dichroism technique to probe the properties of many different chemical species relevant to the pharmaceutical develop-
opment process. While many techniques used in pharmaceutical development are specific to protein-ligand interactions, CD can be used to examine proteins, carbohydrates, nucleic acids, biopolymers, and small ligands. Importantly, the technique can show what happens to mixtures of complex biological molecules in solution and how they might interact and form complexes in nature. This type of interaction is critical in pharmaceutical development, as it helps to show if and how drug candidates interact with their targets and if there are any potential challenges that need to be overcome.

CD is particularly apt for studying chiral materials. For pharmaceutical development, the chirality of drug mixtures, as well as the different biopolymers with which they interact, can have a dramatic effect on the outcome of the interaction. CD can help to determine the link between structure and function on biomolecules, which is essential information to know if a drug candidate is able to have an effect.

When investigating the wealth of information we have about proteins, it is easy to imagine that well-structured and immobile proteins are of the highest importance. These are the types of structures that are represented in the Protein DataBase (PDB). However, in their native state, between one third and one half of mammalian proteins are disordered and are not amenable to immobilization or crystallization. This makes them unsuitable to be studied using methods like crystallography and nuclear magnetic resonance (NMR), but ideally suited for a technique like CD.

The CD beamline at B23 delivers a collimated beam of UV light with a relatively small cross-section of approximately 1 mm × 2 mm that can be focused to about 30 micron × 50 micron for spatially resolved mapping of molecules in solid-state thin films. The small cross-section compared with commercial CD instruments means that much smaller volumes can be used, which is useful for precious samples or experiments that require a higher throughput. Synchrotron light also means that the UV beam has a high flux, which helps to improve the signal-to-noise ratio. While B23 has a wide range of potential uses,
many of the experiments done there have generated data that can be of use for pharmaceutical development.

Acute myeloid leukaemia (AML) is a debilitating and often fatal disease with a rapid onset. It is a particularly insidious form of cancer where diseased cells are able to interfere with the immune system and prevent the body’s natural cancer defenses from working effectively. Research carried out at B23 has aimed to understand how AML cells are able to do this by studying the interaction of AML cell-specific receptors with a natural ligand present on white blood cells and certain epithelial cells. The AML receptor named LPNH1 is present in much higher abundance on the surface of cancer cells when compared to other cell types that naturally possess this receptor. The binding ligand named FLRT3 is naturally present in the blood of healthy individuals, but the interaction with LPNH1 AML cells is a hallmark of the disease. Researchers using B23 found that cortisol was present in the blood of AML patients at much higher concentrations than expected and that cortisol was upregulating the production of the LPNH1 receptor on the surface of AML cells. CD studies were able to confirm that LPNH1 and FLRT3 were incredibly high affinity binding partners, and the molecular interactions were shown. It also became clear that AML cells recruited cortisol, which is a master regulator of human metabolism and can dramatically affect glucose homeostasis and create the perfect environment for cancer cells to thrive.

The upregulation of LPNH1 and the strong binding to FLRT3, lead to some downstream complications that make it impossible for the human body to fight the cancer cells. The interaction of LPNH1 with FLRT3 results in the secretion of galectin-9 and Tim-3, the latter of which protects the cancer cells against immune system attack by natural killer (NK) cells. Using CD, it was possible to define the precise interaction of galectin-9 and Tim-3 and show that conformational changes in solution were essential for the binding of this pair of proteins [1].

CD was then used to investigate this further. A unique complex of galectin-9 and Tim-3 was looked for in a number of different cancer types. The specific formation of these molecules gives a unique signature when using the CD technique. The complex was discovered in breast cancer tissues as well as a whole range of different cancer cell types. This important finding suggested that many cancers were using the galectin-9 Tim-3 complex as a way of defending themselves from the human immune system. The advantage of using CD to carry out the investigation is that now it is possible to use the technique to screen potential drug candidates looking for any ligands or changes in the solution that will break up the galectin-9 Tim-3 complex. Doing this as a therapy could be the key to allow the body’s natural defenses to destroy cancer cells. It was essential to use CD for this application, as it is one of the only techniques that could show the associations between the two molecules in a complex environment [2].

As shown, diseases often involve complex interactions between many different molecular species that are dynamic in nature. CD can help to understand these complexities and provide a more accurate picture of the true dynamic interactions taking place in nature. A study carried out at B23 helped to elucidate the structure of chaperone proteins. Chaperones are specialized proteins that help other proteins to fold properly. The interactions are complex and are often required when cells are undergoing some kind of stress. The chaperone Hsp90 is designated Hsp for Heat Shock Protein, as it was discovered during heat shock exposure to cells. While the existence and function of chaperones have been known for decades, the precise composition of chaperone complexes has eluded scientists for a long time. Chaperone complexes are, in their nature, dynamic. They form and dissociate quickly to allow the proteins to fold in exactly the right way. With such an important function, it is easy to understand that failure of chaperone complexes properly can lead to imbalances in the cell.

CD was used at B23 to understand how a chaperone complex made up of Hsp90, Sgt1, and Rar1 was assembled. While the structure of the three proteins was already known, the stoichiometry was not fully understood. The researchers used the circularly polarized light to differentiate between the different proteins without the need for labels or crystallization, which had been the only way they had previously been characterized. Analysis of the CD data revealed a clear relationship between an Hsp90 dimer, two Sgt1 molecules, and a single Rar1 molecule.

This finding is an important example of how CD can complement MX. The structure of the Hsp90 complex as a crystal had been solved so that it was known how the proteins could associate with one another. However, there was no direct evidence for exactly how the proteins would associate in the cell. Crystallization conditions are often not simulating the cellular environment as each buffer used has to be optimized to produce crystals. Using CD and having prior knowledge of the structures from MX experiments made it possible to understand one of the most important protein complexes in the human body.

The work done on Hsp90 does not immediately suggest a new pharmaceutical product, but it saliently highlights one of the key challenges facing drug discovery going into the future. The problem is that a lot is known about many proteins, but only under a very limited set of extreme conditions. In order to understand disease and be able to discover new treatments, it is essential to use techniques that add new information to what we already know about protein complexes [3].

Experiments using CD at B23 have also been used to develop new ways to kill pathogenic bacteria. As antibiotic resistance becomes an increasing threat, new ways to attack microbes that are resistant to common antibiotics are needed. An ambitious synthetic biology approach aimed to take host proteins that were known to attack the bacterial cell membrane and assemble them as bacterial viruses with deadly spikes protruding out from every angle. The inventive idea requires that the host proteins are able to be manipulated and packed at a specific density on a sphere while remaining active. Nothing that
happens during their processing can affect the antimicrobial properties of the host proteins. CD was used in order to understand exactly how the host proteins and the assembled particle were behaving. The experiments showed that the host proteins remained unfolded in solution, but were ultimately able to attach and fold properly on the spherical surface. The confirmation of this was an important step for the researchers to understand that the antimicrobial capsid had been constructed correctly. It also provided a method for testing how the capsid was affected by the engagement with bacteria, and a method for producing capsids decorated with a whole host of different antimicrobial molecules [4].

While CD experiments have proven to be extremely valuable for pharmaceutical discovery, they are often used further down the development pipeline to ensure that drugs behave exactly as expected or to assess the function and stability of known drug candidates.

A study was designed to try to stabilize proteins that were sensitive to heat. This was to address the broader problem that many pharmaceutical compounds are biological in nature and require a “cold chain” from the moment they are synthesized to the moment they are administered to the patient. Finding ways around this could drastically improve the accessibility of pharmaceutical products.

A research team encased some heat labile proteins in a silica cage inspired by the marine microorganisms diatoms. CD was one of the techniques used to evaluate the viability of the proteins as the silica cage was subjected to heat. The results clearly demonstrated that the proteins were indeed protected and that this new approach could have the potential to decrease the reliance on a cold chain for sensitive biomolecules used as pharmaceutical products [5].

X-ray absorption spectroscopy

XAS is a lesser-known technique for use in pharmaceutical development. However, the method has some unique benefits that are particularly suited to a specific class of pharmaceutical products; namely, those that contain metals. XAS can efficiently detect the oxidation state of metals at the center of drugs or coordinated with protein complexes. However, this is pretty much all they can detect. What seems like a disadvantage can be extremely useful if noisy samples are unable to show what is really happening between a target and a ligand.

B18 (Figure 3) is a bending magnet beamline that can cover an energy range between 2-35 keV. The microfocus means that very small samples can be investigated using XAS, which is an important consideration for precious samples. Samples in B18 can also be measured in solution, which is critical for development of pharmaceuticals, all of which must be in solution when they are eventually used.

Studies using B18 investigated DNA binding molecules that contained platinum and palladium. These molecules were exposed to DNA from breast cancer cells to understand if they could cause some toxicity and kill the cancer cells, demonstrating whether they could be used as therapeutic drugs. The experiments investigated the interaction of two cisplatin-like dinuclear Pt(II) and Pd(II) complexes with cellular DNA. The B18 beamline was used to perform X-ray spectroscopy measurement that could visualize the interaction of the Pt(II) and Pd(II) with DNA and how they affected the DNA’s dynamical profile. Researchers used techniques such as quasi-elastic neutron scattering (QENS) coupled with synchrotron-based extended X-ray absorption fine structure (SR-EXAFS) and Fourier-Transform Infrared Spectroscopy-Attenuated Total Reflectance (SR-FTIR-ATR). This allowed them to see that the DNA profile changed substantially and that the molecules were indeed toxic. This was the first time that these drugs had been observed directly interacting with their target [6].

Ruthenium complexes have also shown promise as anticancer drugs. One particular Ruthenium complex, NKP-1339 (sodium trans-[tetrachloridobis(1H-indazole)ruthenate(III)]), is close to reaching the clinic. While clinical trials have been successful and progressed a long way, the precise mode of action of NKP-1339 is unknown. Previous studies reported that the mode of action was linked to the redox chemistry of Ruthenium; however, the precise redox state and coordination of the atoms had not been clearly shown. Research at B18 used the X-ray Absorption Near Edge Structure (XANES) technique, which provides detailed information about the speciation of metal centers, including
oxidation state and coordination environment. Using the B18 beamline, experiments revealed that chloro ligands remained tightly covalently bound to the Ru ion, a transformation that could only be observed in vivo and could help to explain the effects of NKP-1339 [7].

**Summary**

While MX has been the workhorse of drug discovery for many years, advances in synchrotron methods with complementary properties are proving to be invaluable in the search for new pharmaceutical compounds. CD performed at B23 and X-ray absorption studies have already contributed important information in the development of a range of pharmaceutical drug candidates from cancer to novel antimicrobials.

CD and X-ray absorption are by no means limited to the study of modern-day pharmaceuticals. Material, electronics, and a whole variety of biomixtures can be studied using both of these techniques. As new technologies come online that help patients and medical staff interact with medicine in a different way, CD and XAS will have an important role in developing the drug delivery systems of the future.

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