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The Evolving Role of Structural Biology in Drug Discovery

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Corpora non agunt nisi fixata.
Paul Ehrlich (1854–1915)

In science truth always wins.
Max Perutz (1914–2002)

A picture is worth a thousand words.

1.1 Introduction

Life is based on "informed matter" (matter + information) shaped up by thousands of chemical reactions and intricate interaction networks, and biology primarily relies on the observation of structures, historically from organisms to organs, tissues, cells, organelles and, more recently, macromolecules and macromolecular assemblies, following the progress of observation instruments and techniques. In this book, "structural biology" refers to molecular structural biology, starting in 1953 with the discovery of the double-helical nature of DNA, crucially based on unacknowledged experimental data from Rosalind Franklin, the true hero behind this discovery [1, 2]. However, the real breakthrough, achieved the same year, was the solution to the phase problem by Max Perutz [3], which led to the determination in the late 1950s of the first crystallographic structures of proteins, that of myoglobin at 2 Å [4, 5] and that of hemoglobin at 5.5 Å [6].

Modern drug discovery is a highly complex and multidisciplinary activity that began at the turn of the twentieth century [7], and structural biology started to have an impact in the seventies [8]. A founding father in the field and a visionary, Max Perutz not only opened the way to macromolecular crystallography by solving the phase problem but also understood very early the huge potential of structural biology for drug discovery. In 1968, he could relate clinical symptoms and abnormal properties of mutant hemoglobins with the probable structural alterations caused by the corresponding mutations based on the crystal structure of hemoglobin [9]; in 1976, he reviewed the relevance of molecular biology, and particularly of molecular structural biology, to medicine, predicting that the tridimensional (3D) structures of enzymes could be used to design "tailor-made" inhibitors to treat diseases [10]. Indeed, the same year appeared the first rational ligand design study based on the crystal structure of deoxyhemoglobin in complex with 2,3-diphosphoglycerate [11].

Actually, the first drug derived from a structure-based approach, the antihypertensive captopril [12], an angiotensin-converting enzyme inhibitor, was approved in 1981, followed by dorzolamide [13], a carbonic anhydrase inhibitor used for the treatment of glaucoma, in 1995. The concept of structure-based drug design (SBDD) became popular in the 1980s, but technical bottlenecks hampered its early adoption by the pharmaceutical industry due to the high costs and long timelines involved. The first important breakthrough had been recombinant DNA technology [14], which enabled the production in bacteria of large amounts of proteins for structural studies thanks to heterologous gene expression systems, but early expression systems often failed to produce soluble proteins from mammalian cDNAs, and few protein structures of therapeutic interest were available at the time. In the late 1990s, a series of technical improvements and breakthroughs at all levels, including:

- new recombinant gene expression systems, including affinity tags for efficient protein or multiprotein complex purification,
- parallelization, miniaturization, and automation of gene expression tests; protein purification; and protein crystallization,

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- advances in X-ray crystallography (cryo-cooling, sample changers, micro-focus beamlines, single-photon-counting detectors, seleno-methinonine derivatives for phasing, etc.) and nuclear magnetic resonance (NMR) (high-field magnets, sample changers, cryo-probes, site-specific labeling, etc.),
- progress in software integration, as well as the massive increase in the speed of computers and in data storage capacities,

altogether leading to high-throughput automated data collection and processing, made this adoption possible.

Most of these developments were driven by the structural genomics (or, more correctly, structural proteomics) initiatives [15–17] that followed the genome sequencing projects, particularly the Human Genome Project [18] (see also the "Structural Genomics Supplement" of Nature Structural Biology, November 2000 issue). Structural genomics consortia initially worked on bacterial targets because they were more amenable to a high-throughput approach, but later some of them, such as Structural Proteomics IN Europe (SPINE) and the Structural Genomics Consortium (SGC), focused on human targets, more difficult to tackle but of high potential value for drug discovery programs [19, 20].

In the late 1990s, another important approach, fragment-based drug design (FBDD), reviewed in Chapter 4, was developed in industry, by both big pharmas and small biotechs [16, 21–23], even though the seminal concept by William Jencks in 1981 [24] and the first proposal of fragment cocktail crystallography by Wim Hol in 1990 [25] came from academia. It emerged as an alternative to the then dominant high-throughput screening (HTS) approach, based on massive combinatorial chemistry and looking for hits with nanomolar binding affinities, which in many cases failed to deliver easily optimizable hits. Through the screening of much smaller libraries (10^2–10^3) of smaller compounds called fragments (carefully selected compounds with a molecular mass around 200 Da) compared with the huge libraries (10^5–10^6) of "lead-like" and "drug-like" compounds with a molecular mass around 500 Da built for HTS, the chemical space is more efficiently sampled [26], and better starting points for the design of lead compounds are identified [27, 28]. Since the idea is to look for fragment hits binding to "hot spots" on the target even with millimolar affinities that can then be grown, linked, or merged into leads with much higher affinities, the rise of FBDD was allowed by the development of sensitive, high-throughput biophysical techniques for fragment screening [29] and the careful design of synthetically useful fragment libraries [30]. FBDD has proved to be effective in accelerating the hit-to-lead process, two fragment-derived drugs have already been marketed (the selective B-Raf V600E inhibitor vemurafenib in 2011 for the treatment of metastatic melanoma – only six years from project inception to market – and the Bcl-2 inhibitor venetoclax in 2016 for the treatment of chronic lymphocytic leukemia), and dozens of compounds are currently in clinical trials [31].

Now structural biology has been fully integrated in the pharmaceutical industry drug discovery pipeline, and many companies use both HTS and fragment screening to identify starting points for their small-molecule R&D programs. As HTS is still much used [32] even though it is labor intensive and costly, combining HTS with FBDD allows to get the best of both worlds, for instance, through deconstruction/reconstruction strategies [33] that can help in lead optimization, in particular by suggesting ideas for scaffold hopping. This has to be considered in a larger context where drug discovery in big pharmas tends to be more and more highly automated [34]. Hence the requirement, for structural biology to significantly impact a drug discovery program, that the structure of a newly validated target be available right from the start with a robust system (mostly based on X-ray crystallography up to now) already set up to allow the high-throughput determination of hundreds of target–ligand complex structures.

For an optimal therapeutic effect, a drug should have a known mechanism of action. In the old days, drugs were developed from "black-box" phenotypic screenings using biological assays, mainly cellular or animal models related to pathological states, but the attrition rate was high due to a poor knowledge of the human biology underlying diseases. Progress in biochemistry and molecular biology has triggered a shift toward target-based drug discovery, boosted by the advent of high-throughput genomics and proteomics technologies and the completion of human genome sequencing [35]. The target-based approach, to which SBDD applied to small molecules is ideally suited, has been dominant for the three past decades. However, this approach also failed to significantly decrease attrition in clinical phases, mostly due to lack of efficiency, which can be explained by the reductionist character of the "one disease–one target–one drug" paradigm and often by insufficient validation of the target [36]. This is why there has been in the recent years a renaissance of phenotypic drug discovery [37, 38] with powerful new tools such as high-content screening, ultimately leading to identification of new targets, for instance, through the use of chemogenics libraries for which both the target and the mechanism of action are known [39].

Classically, proteins have essentially been the targets of small-molecule drugs, but, with the advent of biologics (also called biotherapeutics or biopharmaceuticals), they have also become drugs themselves, including

hormones, enzymes, and antibodies, and the structural biology methods were already available to characterize and optimize them. Already in 1972, the biological properties of chemically modified insulins were discussed in relation to structure [40]. Insulin started to be used as a drug in the early 1920s, but it was purified from porcine or bovine pancreas. The recombinant human insulin, the first "biologic" in the modern sense, was marketed in 1982 by Eli Lilly. Since then, the number of biologics has been steadily increasing, reaching 38% of the R&D pipeline drugs in 2017, mostly antibodies and other recombinant proteins [41]. In 2017, among the top 10 drug worldwide sales, 8 were biologics including 6 antibodies, 1 protein conjugate vaccine, and 1 recombinant insulin analog [42]. However, among the newly approved therapies over the 2010–2017 period, the small-molecules-to-biologics ratio has remained fairly constant (~75%/25%) [41], suggesting that the importance of biotherapeutics will certainly continue to increase but without putting an end to small-molecule drugs, at least for a near future.

Now biologics are diversifying, involving types of macromolecules such as RNA [43] or DNA (for gene therapy), and are becoming more complex, e.g. CRISPR/Cas genome editing systems [44] and cell-based therapies.

Molecular recognition between a target and a ligand is a central theme of structural biology in drug discovery, starting from Paul Ehrlich's statement "Corpora non agunt nisi fixata." Modern SBDD relies not only on structural information but also on information on the thermodynamics, kinetics, and dynamics of target–ligand interactions, hence the inclusion of complementary information from various biophysical techniques as well as molecular dynamics simulations. Chapter 5 summarizes how thermodynamic and kinetic information can be used in SBDD. For a long time, SBDD had mostly relied on the essentially static information from crystal structures and on dynamic information from NMR. Target–ligand interaction models have evolved from a static to a dynamic vision and from lock-and-key to induced fit to conformational selection and ligand binding ensembles, and protein flexibility is now recognized as a crucial parameter for protein function and is more and more taken into account for drug design (as well as ligand flexibility) thanks to ever-increasing computing power. Allostery [45–47] has become an important concept in drug design and is discussed in Chapter 6.

1.2 The Expanding Toolbox of Structural Biology for Drug Discovery

The precise knowledge of the structure of cellular macromolecules (proteins and nucleic acids), their stable or transient assemblies (multi-protein complexes and nucleoprotein complexes), and their complexes with lipids, cofactors, and ligands (endogenous, dietary, or xenobiotic) is essential to understand their function, in particular their dysfunction in disease, and to design therapeutic strategies aiming at directly or indirectly modulating their activity by drugs. X-ray crystallography and NMR have long been the most used structural biology techniques in drug discovery, but the toolbox has expanded with the increasing role of biophysics [29], the use of a whole range of computational techniques ranging from docking to molecular dynamics ([48] and Chapter 15), and the recent burst of cryo-electron microscopy (cryo-EM) thanks to the so-called resolution revolution [49, 50]. Now structural biology has become instrumental in target-based/mechanism-based drug discovery, and Part II of the book reviews the main techniques used in structural biology in a drug discovery context. The direct imaging techniques used in structural biology are shown in Table 1.1.

To obtain atomic-scale information on target–ligand complexes, even though atomic resolution (by the way, a term to be used with caution [51, 52]) is rarely achieved, the main direct imaging technique that has been used is single-crystal X-ray crystallography, mostly at synchrotrons. It is not the purpose of this book to describe in detail the basics and various aspects of macromolecular X-ray crystallography, a huge field in itself, and readers who want more comprehensive information on this topic are encouraged to refer to a selection of outstanding books [53–55]. High-throughput crystallography allowed by extensive automation at third-generation synchrotron radiation sources [56–60], optimized for high brilliance, has matched the needs of industrial programs [15, 16, 61]. However, a number of limitations were still remaining, and some of them have been addressed by recent developments in the field. For instance, *in situ* diffraction [62–65] and automated crystal harvesting [66–68] have alleviated the burden of handling and mounting crystals. Microcrystallography involving micro-focus beams 20 µm or less in diameter [69] allowed microcrystals (10–50 µm) or imperfect crystals to be used for data collection, circumventing the problem of growing large crystals for challenging targets including membrane proteins. Even so, radiation damage remains an important issue although it is significantly reduced by cryo-cooling [70, 71], but it can be eliminated with serial femtosecond crystallography (SFX), a radically new approach that uses an X-ray free-electron laser (XFEL) facility as a source of femtosecond X-ray pulses [72–75]. Nanocrystals are brought to the beam either via the continuous stream of a viscous medium thanks to an injection device or on a fixed target that is rastered by the beam and are never shot more than once by an X-ray pulse, leading to room-temperature, radiation-damage-free structures.

Table 1.1 Direct imaging techniques used in drug discovery.

Sample	Probe	Technique	Interaction	Outcome	Maximum resolution obtained with macromolecules
Solid (crystal)	Photons (X-rays)	X-ray crystallography	Electrons	Electron density map	~0.5 Å
Solid (crystal)	Electrons	Electron crystallography (including micro-electron diffraction [MicroED])	Electrons and protons	Electrostatic potential map	~1 Å
Solid (crystal)	Neutrons	Neutron crystallography	Nuclei	"Nuclear density" map	~1 Å
Solid (cryo-cooled solution)	Electrons	Cryo-electron microscopy (cryo-EM)	Electrons and protons	Electrostatic potential map	~1.5 Å
Solution	Photons (X-rays)	Small-angle X-ray scattering	Electrons	Molecular envelope	~1 nm
Solution	Neutrons	Small-angle neutron scattering	Nuclei	Molecular envelope	~1 nm
Cryo-cooled cell suspension	Electrons	Electron tomography	Electrons and protons	Electrostatic potential map	~5 nm
Cryo-cooled cell suspension	Photons (X-rays)	Soft X-ray tomography	Electrons	Electron density map	~25 nm

The "diffract before destroy" concept behind XFEL was put forward in 1986 [76], simulated in 2000 [77], and experimentally established in 2011 [72]. SFX has been most successful for structure determination of membrane proteins grown in a lipidic cubic phase (LCP) as both the crystallization and sample delivery medium [74, 75] and also enables ultrafast time-resolved studies ("molecular movies") [78–80]. SFX also provides more native structures since working at room temperature avoids the bias of conformational trapping at very low temperature, as is the case with cryo-cooling. Pioneers in the field describe in Chapter 27 the historical landmarks of the use of XFELs in structural biology and the remaining challenges. And finally, the spectacular success of SFX prompted synchrotron scientists to combine the use of a micro-focus beamline with a rapid detector and microcrystals either on a fixed target or in the continuous flow of a microcrystal suspension to perform multi-crystal data collection, leading to serial microcrystallography (SMX) at synchrotron facilities, also called serial synchrotron crystallography (SSX) [81, 82]. In this way, radiation damage is minimized, even at room temperature [83].

Chapter 10 provides an exhaustive account of these recent technical advances in X-ray crystallography as well as an outstanding integrated vision of the whole process from crystals to diffraction data to electron density map to model and how it should be optimally implemented in the future to address the high-throughput imperative of the pharmaceutical industry.

Neutron crystallography (Chapter 12) has also been used, albeit marginally, because of the paucity of neutron sources and the need for large crystals (~mm^3) to compensate for the low flux of available neutron beams, even though the new spallation sources produce much more intense beams allowing for much shorter collection times and the use of smaller crystals [84, 85]. However, neutron crystallography brings complementary, sometimes crucial information on the position of hydrogen atoms, in particular the protonation state of the ligand, of specific amino acids of the target, and of water molecules in the active site. Indeed, the reversible binding of protons plays a key role in the catalytic mechanism of many enzymes and in allosteric regulation. Neutrons interact with nuclei, and the scattering lengths of hydrogen (and deuterium) nuclei are in the same range of magnitude as those of other atoms; hence the H/D atoms are clearly seen in "nuclear density" maps (although it is slightly more complicated for hydrogen due to its negative scattering length, hence the widespread use of H/D exchange). On the other hand, X-rays are scattered by electrons, and thus X-ray scattering depends on the atomic number Z, and hydrogen atoms are hardly seen in X-ray crystallographic structures, except (partially) at ultrahigh resolution. Even though the position of most hydrogen atoms can be inferred from the positions of the other atoms from stereochemical considerations (sometimes only statistically due to the rotational degrees of freedom), some hydrogen atoms cannot be unambiguously located, for instance on

histidine, aspartate, and glutamate residues. For instance, it is sometimes essential to have a complete knowledge of an enzymatic catalytic mechanism in order to design an efficient inhibitor, and in such cases neutron crystallography is a powerful complement to X-ray crystallography, as shown by recent joint X-ray/neutron crystallographic studies for drug design (see, for instance, [86–88]). Another advantage of neutron crystallography is the lack of radiation damage to crystals even at room temperature with the beams used for diffraction experiments.

The third crystallographic technique used in structural biology is electron crystallography. It was used in the early days of electron microscopy to obtain structures from 2D crystals [89–92], but 2D crystals are not always available, and its use declined with the development of single-particle analysis (SPA) cryo-EM (see below). Electron crystallography has recently reemerged thanks to a new electron diffraction technique called MicroED that uses 3D microcrystals [93, 94].

For all crystallographic studies (using X-rays, neutrons, or electrons), the first and obligatory step is to obtain crystals, hence the importance of the science of macromolecular crystallization (see part 4 of [54] as well as [95–99]). Industrial aspects of target–ligand crystallization campaigns are described in Chapter 8. Crystallization of membrane proteins is especially challenging and is described separately in Chapter 9; as it will certainly remain a real bottleneck for crystallographic structure determination, in this domain cryo-EM will certainly play a very important complementary role.

By the way, it is of utmost importance that all macromolecules or molecular complexes that are to be used for crystallization trials as well as for almost all structural or biophysical studies be of the highest possible purity and their quality (identity, conformational homogeneity, oligomeric state, stability in terms of oxidation and aggregation, characterization of contaminants, etc.) be biophysically assessed through a thorough quality control protocol, as described in Chapter 7. This is even more crucial when the macromolecule is a biotherapeutic drug candidate, as illustrated in Chapter 21 for the characterization of therapeutic antibodies by mass spectrometry techniques. When the macromolecule or molecular complex is the target, it has been shown that the use of biophysical methods increases the success in obtaining target–ligand complex crystal structures [100].

A major recent evolution in structural biology has been the advent of cryo-EM as another major tool for the high-resolution structure determination of macromolecular complexes. As neutron crystallography, electron microscopy is a fallout of the wave–particle duality hypothesis formulated in 1924 by Louis de Broglie in his PhD thesis [101] – hence the need to support basic science. The first electron microscope was built by Ernst Ruska and Max Knoll in 1931, and since then the limit in resolution has been regularly pushed back by progress in technology (accelerating voltage, electron optical lenses, vacuum systems, etc.). But radiation damage was a bottleneck until the invention of cryo-EM in the early 1980s [102]. However, for a long time, its use was restricted to the study of large complexes that could not be crystallized and usually led to low-resolution models. The most significant breakthrough in the recent years has certainly been the spectacular progress of SPA cryo-EM. Recent improvements in microscopes, in sample preparation and vitrification, and in software (object classification, projection image alignment) as well as the introduction of single-electron detectors and Volta phase plates have led to cross the 3-Å resolution limit and to study smaller objects (see, for instance, [103–105] and Chapter 26). SPA cryo-EM has already made and will continue to make a huge impact in biology by enabling the structural study of a number of biologically important, large, and/or dynamic assemblies that cannot be crystallized, including complexes that undergo large conformational changes such as the spliceosome. Besides, the role of cryo-EM in drug discovery has also been discussed recently [106–111]; although there is clearly a huge potential ahead, it appears that at present cryo-EM is not yet ready to be used as a routine tool for SBDD. Important barriers have already started to be broken down, namely, in terms of resolution achieved and size of study objects ([103] and table 1 of [111]), but major bottlenecks such as sample vitrification, throughput of data collection, and access to microscopes have to be overcome for cryo-EM to compete with high-throughput X-ray crystallography. Robust automated workflows with rigorous criteria to assess the quality of cryo-EM maps and structures still need to be set up.

The other main experimental technique to build atomic models of macromolecules and their complexes is NMR, although it is not a direct imaging technique but rather a spectroscopic technique. NMR yields detailed structural information from the chemical environment of atomic nuclei and the interactions between them. Moreover, NMR gives access to the dynamics of molecular systems in solution. It is also a versatile and sensitive tool to study target–ligand interactions. Due to its importance, two chapters are dedicated to NMR: Chapter 13 describes its use for the determination of protein structure and dynamics, which is crucial for the characterization of biotherapeutics and for the study of challenging targets such as intrinsically disordered proteins (IDPs) or disordered protein domains that cannot be crystallized (see also Chapter 25), and Chapter 14 describes the use of NMR studies of target–small molecule interactions in drug discovery.

Table 1.2 recapitulates key events in the development of the three main techniques (X-ray crystallography,

Table 1.2 Selected key events in the development of the three main techniques (X-ray crystallography, NMR, and cryo-EM) used for 3D structure determination of macromolecules and their adoption for drug discovery by the pharmaceutical industry.

Field	Year	Event	Associated Nobel Prize
X-ray crystallography	1953	Max Perutz solved the phase problem [3]	
	1958–1960	Crystal structures of myoglobin (Kendrew) [4, 5] and hemoglobin (Perutz) [6]	Max Ferdinand Perutz and John Cowdery Kendrew (1962)
	1969	Structure of insulin [112]	Dorothy Crowfoot Hodgkin (1964)
	1970s	Structures of globins, enzymes, and polypeptide hormones	
	1971	Protein Data Bank [113]	
	1979	Collaborative Computational Project No. 4 (CCP4) – Software for Macromolecular X-Ray Crystallography [114]	
	1981	Approval of Capoten™ (captopril), first drug derived from a structure-based approach (hypothetical model of the target, ACE, based on the crystal structure of carboxypeptidase A, a mechanistic homolog) [12]	
	1985	First X-ray crystallographic determination of a membrane protein structure [115]	Johann Deisenhofer, Robert Huber, and Harmut Michel (1988)
	1989	Crystal structures of viral proteases [116–120]	
	1994	Structure of F1-ATPase [121]	John Walker (1997)
	1995	Approval of Trusopt™ (dorzolamide), first drug unequivocally derived from structure-based drug design (SBDD) [13]	
	1998	First structure of an ion channel [122]	McKinnon (2003)
	2000	First structure of a mammalian GPCR [123]	
	2000s	High-throughput X-ray crystallography at synchrotron beamlines: sample changers, automated operation, and remote data collection [56–60]	
	2003	40+ compounds derived from SBDD have entered clinical trials; 7 of them have been approved [124]	
	2011	Refined structure of the yeast ribosome, largest asymmetric macromolecular assembly for which a crystal structure was determined so far [125]	
	2011	Structure of a GPCR – G protein complex [126]	Brian K. Kobilka (2012)
	2011	Approval of vemurafenib, first drug derived from FBDD [28, 127]	
NMR	1946	Invention of nuclear magnetic resonance [128, 129]	Felix Bloch and Edwards Mills Purcell (1952)
	1957	First spectrum of bovine pancreatic ribonuclease at 40 MHz [130]	
	1966	Application of Fourier transform spectroscopy to magnetic resonance [131, 132]	Richard Ernst (1991)
	1971	2D NMR introduction [133–135]	
	1985	Proteinase inhibitor IIA solution structure by ^{1}H NMR [136]	Kurt Wüthrich (2002)
	1988	3D NMR introduction [137, 138]	
	1996	First proof of concept for FBDD: "SAR by NMR" [21]	
	1999	Saturation transfer difference (STD) NMR [139]	
	2000	Screening by NMR against proteins containing 13C-labeled methyl groups [140]	

Table 1.2 (Continued)

Field	Year	Event	Associated Nobel Prize
	2000	Screening by NMR based on magnetization transfer from bulk water (WaterLOGSY) [141]	
	2000–2002	Spin labels to identify protein–ligand interactions by NMR [142]	
	2002	"NMR reporter screening" for the detection of high-affinity ligands [143]	
	2003	High-throughput NMR screening using fluorine (FAXS) [144]	
	2004	Affinity-tag-based NMR screening of protein–protein interactions [145]	
	2010	NMR sample changer for medium-throughput automation [146]	
Cryo-EM	1968	3D reconstruction of the helical tail of bacteriophage T4 by electron crystallography [89]	Aaron Klug (1982)
	1981	Cryo-electron microscopy of thin vitrified specimens [102, 147, 148]	Jacques Dubochet (2017)
	1981	Use of multivariate statistics in analyzing the images of biological macromolecules [149]	Joachim Frank (2017)
	1990	Structure of bacteriorhodopsin by electron crystallography [91]	Richard Henderson (2017)
	1995	In theory, 10 000 images of particles are sufficient to provide an atomic-resolution structure of a 100-kDa complex with EM [150]	Richard Henderson (2017)
	1996	Refined structure of bacteriorhodopsin by electron crystallography [151]	Richard Henderson (2017)
	2002	Electron Microscopy Data Bank (EMDB) [152]	
	2005	Structure of aquaporin 0 at 1.9 Å by electron crystallography reveals lipid–protein interactions [153]	
	2007–2008	First structures of high-symmetry viral particles at near-atomic resolution [154–156]	
	2008	First structure of a non-icosahedral particle at near-atomic structure [157]	
	2009	Fully automated 12-sample changer [158, 159]	
	2011–2013	Direct electron detector introduced, with "movie" mode [160, 161]	
	2012	Launching of Collaborative Computational Project for Electron Microscopy (CCP-EM) [162]	
	2012	Proteasome: first structure of a mega-complex solved by a combination of cryo-EM and X-ray crystallography [163]	
	2013–2014	Structures at near-atomic resolution by cryo-EM [161, 164–166]	
	2014	Volta phase plates introduced [167]	
	2017	Structures of GPCR – G protein complexes at near-atomic resolution [168, 169]	
	2017	Rabbit muscle aldolase structure at 2.6 Å solved by single-particle cryo-EM at 200 keV [170]	
Orthogonal techniques	1969	Transfection technology [171]	
	1973–1974	Recombinant DNA [14]	
	1980s	Structural bioinformatics (molecular modeling and computational chemistry)	
	1981	Fragment-based drug design (FBDD) conceptualized by William Jencks [24]	

Source: Adapted from figure 2 of [111].

NMR, and cryo-EM) used for 3D structure determination of macromolecules and their adoption for drug discovery by the pharmaceutical industry (adapted from [111]). Concerning X-ray crystallography, Tom Blundell, another pioneer in the field, has given a vivid account of the exchanges between academia and industry over the whole period [172].

As an experimental structure is always a model built from experimental data, it always relies in part on interpretation, and structural information (in practice, mostly derived from X-ray crystallography) should always been analyzed with a critical eye and used with caution in drug design [173, 174] – hence the crucial need for thorough validation process, as explained in detail in Chapter 11 [175].

X-ray crystallography maps show electron densities and thus somehow represent the chemical identity of atoms (although this begins to appear only at ultrahigh resolution). However, these maps may be biased since they are computed from both measured intensities (including experimental errors) and estimated or calculated phases that also include errors; moreover, a frequent source of problems is density overfitting.

Concerning cryo-EM, one should bear in mind that it provides electrostatic potential maps (thus not directly comparable with electron density maps derived from X-ray diffraction data) that should also be interpreted with caution [176]. Furthermore, concerns have recently been raised about the lack of a thorough validation protocol for cryo-EM structures compared with X-ray crystallography, emphasizing the need for more validation tools [177, 178] and stricter cryo-EM structure deposition standards [179].

Chapter 15 serves as an introduction to computational structural biology/structural bioinformatics, a huge domain that is beyond the scope of this book but is nevertheless an essential component of structural biology for drug discovery.

Finally, some techniques are not described in detail in this book but have been reviewed elsewhere, such as small-angle X-ray scattering (SAXS) and small-angle neutron scattering (SANS) [180, 181] and solid-state NMR [182]. Indeed both X-rays and neutrons can also be used to study macromolecules in solution (Table 1.1). SAXS and SANS are low-resolution techniques, but they usefully complement high-resolution structure analysis by bringing information on the shape and dynamics of large complexes and of IDPs.

A number of established and emerging complementary biophysical techniques are used to study target–ligand interactions (see tables 1 and 2 in [29] and references therein), either in a high-throughput fashion to screen large libraries or with a lower throughput to obtain detailed information on the binding of selected compounds. For screening purposes, the most popular techniques these days are surface plasmon resonance (SPR); differential scanning fluorimetry (DSF), also called "thermal shift assay"; and microscale thermophoresis (MST). Techniques with a lower throughput – but, conversely, with a higher information content – include isothermal titration calorimetry (ITC) and non-covalent/native mass spectrometry. Some techniques are able to probe conformational changes, such as second-harmonic generation (SHG), surface acoustic waves, electrically switchable nanolevers, and, more recently, attenuated total reflection Fourier transform infrared (ATR-FTIR) spectroscopy [183]. Some recent books [184, 185], review articles [29, 186], and a specific conference [187] are dedicated to the growing use of biophysics in drug discovery.

In summary, the increasing use of integrative structural biology in drug discovery implies the need for a comprehensive toolbox: in general one technique cannot bring all answers, and thus a combination of complementary techniques must be used, each having its own limitations and shortcomings. It is also very important to use orthogonal techniques based on different physical principles in order to cross-validate results, which considerably reduces the number of false positives and thus helps to save time and efforts.

1.3 The Various Uses of Structural Biology in Drug Discovery

The basic task of molecular structural biology has been to provide information on the 3D structures and conformational dynamics of the main chemical players of life: macromolecules (proteins and nucleic acids), alone or in complex with natural or synthetic ligands or in more complex assemblies. The Protein Data Bank (PDB) has been a repository for such structures [188], and, since it was established in 1971, ~156 000 structures have been deposited in the PDB, including ~139 000 by X-ray crystallography (~89.1%), ~12 800 by NMR (~8.2%), and ~3 800 by electron microscopy (~2.4%) (figures as of September 2019; for an updated breakdown, see https://www.rcsb.org/pdb/statistics/holdings.do).

This structural knowledge provides the basis to elucidate biological mechanisms at the molecular level and propose new therapeutic modes of intervention/modulation and for rational drug design. Indeed a deep understanding of biological mechanisms underlying pathologies is key to successful drug discovery [189]. Today, structural biology is useful throughout the early-stage drug discovery pipeline (from target identification to lead optimization), but, in the early days, its role was

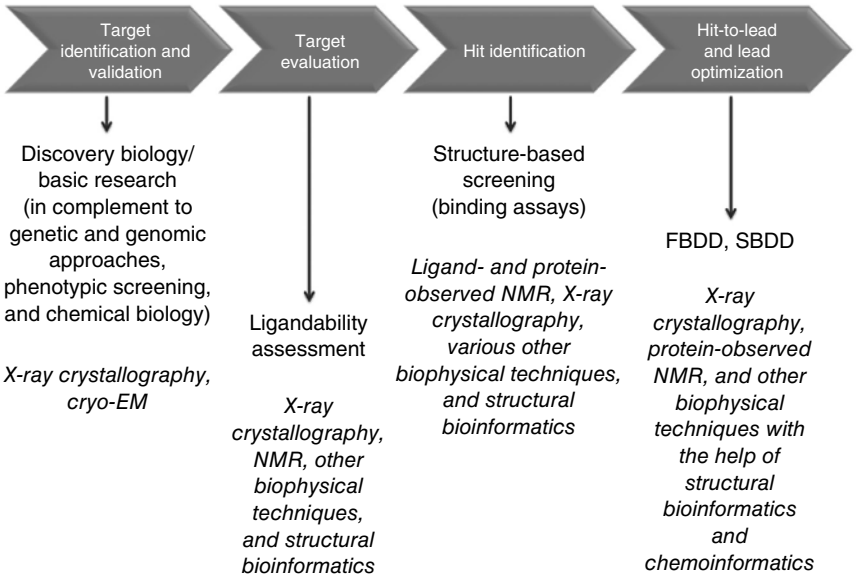


Figure 1.1 Various uses of structural biology in the drug discovery pipeline. Cryo-EM, cryo-electron microscopy; NMR, nuclear magnetic resonance.

essentially confined to the last stage, lead optimization – the classical SBDD approach to improve compound potency and selectivity. Figure 1.1 summarizes the various uses of structural biology in drug discovery.

Indeed, in the 1980s, crystal structures of the target (or of a homolog when the structure of the target itself was not available) started to be used for lead optimization, but, because of the time needed to solve crystal structures, structural biology could not be used at the upstream stages. One of the first reviews on the early attempts to use structural biology for rational drug design dates back to 1986 [190]. Since then, SBDD has become a very powerful strategy to assist medicinal chemists in delivering optimized leads from selected validated hits, essentially by improving the affinity and selectivity of a ligand for a target from the visualization of target–ligand interactions, guided by the progress in biological activity, through iterative (structure → design → synthesis → test) cycles until some desired criteria (e.g. affinity/efficacy/potency and various pharmacological parameters) are reached. There are two major requirements for this approach to be successfully applied:

1) The 3D structure of the target must be available at the start of the project – meaning that the target is known (target-centric drug discovery).
2) A robust crystallographic pipeline must have been set up to progress rapidly.

Indeed, the principles of SBDD were already set in the 1980s, but it was only after several bottlenecks in throughput were overcome thanks to:

- the streamlining of the gene-to-crystal pipeline,
- automated data collection at synchrotron (the crystal-to-diffraction data pipeline),
- automated structure determination and ligand building (the diffraction data-to-structure pipeline),
- progress in computers (computing speed and data storage capacities),

that SBDD could be implemented in pharma companies.

High-throughput X-ray crystallography could then be used not only for lead optimization but also for the upstream stages of the drug discovery pipeline (Figure 1.1):

i) Target identification and validation (discovery biology): as a major tool of structural proteomics and in complement to genetic and genomic approaches, phenotypic screenings (a beautiful example in the case of PCSK9, a challenging target, is given in [191, 192]), and chemical biology that all generate data and hypotheses that need to be validated, which can lead to new ideas for therapeutic intervention. Chemical probes [193, 194] are a great example of the intricacy of structural biology and medicinal chemistry in drug discovery: structural biology can help to design highly potent and selective chemical probes that in turn are used for target validation. Once a target is validated, structural biologists and medicinal chemists continue to work hand in hand to deliver even more potent and selective compounds with drug-like properties. It also happens that a higher-quality chemical probe (thanks to structure-based optimization) leads to the de-validation of a target [195]. Availability of crystal structures also enables structure-based filtering or virtual screening for target deorphanization, e.g. in the case of nuclear receptors [196] or G protein-coupled receptors (GPCRs) [197, 198]. Hits can then be validated for receptor binding by X-ray crystallography or other biophysical methods, optimized through structural studies,

and the resulting chemical tools used to explore biology. SPA cryo-EM has also become an invaluable technique for unveiling new structures of challenging targets and large/dynamic macromolecular complexes as seen from the recent deluge of exciting publications (see, for instance, table 1 of [111]; since 2002, cryo-EM structures are deposited in the Electron Microscopy Data Bank [EMDB] [152, 199]), uncovering new biology and potentially leading to new therapeutic strategies (see, for instance, [200–202]).

ii) Target evaluation (ligandability/druggability assessment), where experimental methods complement sequence-related computational methods and structural bioinformatics [203], as described in detail in Chapter 2, in order to assess at a very early stage whether a target can be modulated by small molecules with drug-like properties, which is key for target prioritization, and to increase the success rate of drug discovery projects.

iii) Hit identification (e.g. fragment screening using X-ray crystallography [22, 204]). Numerous biophysical techniques are used to probe target–ligand interactions (see, for instance, table 1 of [29]), but the great advantage of X-ray crystallography screening is that not only binding is confirmed when a ligand is seen in the electron density map, as well as the identity of the ligand (and hence the occurrence of false positives is minimized), leading to direct deconvolution when compound cocktails are used, but also the precise binding site is revealed as well as the details of the interaction, enabling hit prioritization and constituting the starting point for hit-to-lead optimization. Crystallography has even been used as an affinity technique to isolate and identify strong binders from partially purified natural extracts [205].

iv) Hit-to-lead (typically the FBDD approach), where high-throughput determination of target–ligand complex crystal structures greatly accelerates the fragment-to-lead process.

Structural bioinformatics (Chapter 15) is an essential complement to experimental methods, which can be used, for instance:

- to help introduce selectivity by discriminating the target from close relatives that should not be targeted [206],
- to predict off-targets that may cause adverse side effects [207, 208],
- to design covalent drugs from high-affinity non-covalent ligands [209].

When the protein of interest is no longer the TARGET, as in the case of small-molecule drugs, but the DRUG itself, as in the case of biotherapeutics (including monoclonal antibodies, recombinant proteins, peptides, etc.), structural biology techniques and other biophysical techniques are also used all along the R&D pipeline but with different goals: discovery biology, biotherapeutics selection, biotherapeutics structure determination (see, for instance, the use of NMR in Chapter 13), ideally in complex with its target [210], biotherapeutics characterization and optimization (see, for instance, the use of mass spectrometry for the characterization of therapeutic antibodies in Chapter 21), biotherapeutics quality control (essentially to assess its stability [211] and the presence of contaminants), and biosimilar analysis.

1.4 Evolving Drugs and Targets

In the old days, drugs were mostly derived from ancestral knowledge on plants and, to a lesser extent, minerals. With the development of organic chemistry in the second half of the nineteenth century, natural products started to be synthesized, and many new synthetic compounds became available for pharmaceutical investigations. However, precise mechanisms of action could be revealed only with the advent of biochemistry and later molecular biology and structural biology. For instance, the industrial production of aspirin was started by Bayer at the end of the nineteenth century, but its target, cyclooxygenase (COX), was only isolated in 1976, its cDNA cloned in 1988, and its crystal structure solved in 1994. But in 1991, an alternative form of COX, COX-2, was identified, and in the following years, it was shown that inhibition of COX-2 led to the anti-inflammatory effect of aspirin, while inhibition of COX-1 caused its unwanted side effects. The determination of the crystal structure of COX-2 in 1996 paved the way to the design of COX-2 selective inhibitors with reduced side effects [212].

Today's drugs and drug candidates have become much more diverse and complex, ranging from small molecules to macrocycles, peptides or peptidomimetics, recombinant proteins (including antibodies and their numerous modified versions, such as antibody–drug conjugates [ADCs]), RNAs and their chemically modified versions (aptamers, small interfering RNAs [siRNAs], antisense oligonucleotides [called ASOs or AONs], or mRNAs), DNAs (for gene therapy), and even cells (cell therapy) in order to address more and more challenging targets [213, 214].

In parallel, the range of drug targets has recently considerably widened. New therapeutic candidates are developed that not only act directly on a protein (active-site-directed or allosteric inhibitors, activators, or modulators) or a protein–protein interface (protein–protein interaction inhibitors or stabilizers) but also modulate the expression of a protein target's encoding gene at the level of DNA (genome editing) or mRNA (gene silencing, exon skipping, complementation, inhibition of translation, etc.) or even at

the level of its transcriptional or translational regulation (modulation of gene expression regulatory factors). A recent strategy, targeted protein degradation, consists in specifically inducing the degradation of an unwanted protein thanks to bifunctional ligands often called PROTACs (proteolysis-targeting chimeras) that simultaneously recruit the target protein and a ubiquitin E3 ligase, resulting in the ubiquitination of the target protein, tagging it for proteasome degradation [215].

SBDD is inherently linked to the target-centric approach. At the beginning, SBDD projects were focusing on single pharmacological targets, essentially proteins, that were known or thought to be involved in diseases. The number of existing drug targets has been a recurrent question [216–218]. Two important criteria for pursuing a drug target are its druggability (Chapter 2) and the possibility to design selective drugs that do not bind closely related proteins to avoid potential adverse effects (indeed many protein targets belong to multimember families). Another obvious criterion is target tractability, and the increase in the effective number of drug targets in drug discovery projects using SBDD has paralleled technical progress in protein biochemistry and protein crystallization. Before the 2000s, SBDD was applied mostly to soluble proteins or protein domains. The first crystal structure of a membrane protein appeared in 1985 [115], but the number of membrane protein crystal structures began to grow significantly only after 2000 [219, 220], especially in the therapeutically important GPCR family. Recently, cryo-EM has significantly contributed to the determination of new structures of challenging membrane proteins, including ion channels, and of large membrane protein complexes [111, 221].

The use of structural biology in drug discovery is illustrated in Part III of the book for a selection of important therapeutic target families that have already been intensively studied but still remain challenging:

- Kinases (Chapter 16)
- Serine proteinases (Chapter 17)
- Epigenetic targets (Chapter 18)
- GPCRs (Chapter 19)
- Protein–protein interfaces (Chapter 20)

Finally, the idea that modulating a single target is sometimes insufficient to treat complex diseases opened the way to polypharmacology, also called systems pharmacology or network pharmacology [222, 223]. Chapter 3 describes the *in silico* methods of structural chemogenomics used in the multi-target approach.

1.5 Current Trends and Perspectives

In the swirl around the spectacular progress in cryo-EM, a news feature entitled "The revolution will not be crystallized" suggested crystallography would soon be ousted by cryo-EM [224], but this statement was certainly premature. Indeed X-ray crystallography is far from obsolete [225] and, on the contrary, is constantly reinventing itself, with *in situ* crystallography, automated crystal harvesting, SFX, and SMX, as brilliantly exposed in Chapter 10, and will certainly remain, for a couple of years at least, the most suitable technique for high-throughput SBDD, especially with small-size proteins or protein domains and with fragments. Most probably, both techniques will remain complementary for a long time, and researchers will simply have to choose the one best suited to bring them the information they need at a given stage.

As crystallization is still the limiting step for crystallographic studies, further progress is still needed in the automation [226] and miniaturization of the process [227] and in the follow-up (crystal recognition) [228]. Innovative methods are always needed [74, 229], and future developments should help to address the current and upcoming needs ranging from nanocrystals for SFX and MicroED to large crystals for neutron crystallography [74, 230]. Some recent studies use *in vivo* grown microcrystals for SFX [231, 232] or SMX [81, 233], in particular for direct *in cellulo* diffraction [232, 233].

Cryo-EM will be a most useful complementary technique to answer biological questions, mostly by addressing large and/or dynamic macromolecular complexes and challenging targets such as membrane proteins. Meanwhile, it will undoubtedly continue to make the necessary progress in terms of resolution and throughput needed to become a routine tool in drug discovery. One important issue is the definition of widely accepted, rigorous deposition standards.

MicroED apparently holds a great potential [234, 235] but still has to demonstrate its wide applicability to macromolecule structure determination.

Structural biology is becoming more and more integrated, from atoms to organisms and with the adjunction of the time dimension (4D). As a first step, bridging the gap between molecular and cellular structural biology [236] will not only advance basic knowledge in biology but also give some hints to develop new therapeutic approaches.

Besides continuing to provide 3D – and more and more 4D – information to advance knowledge in biology, structural biology is certainly heading toward very exciting future developments, in particular thanks to the almost infinite possibilities opened by the so-called new modalities [213, 214]. More than ever structural biologists will work hand in hand with medicinal chemists (see, for instance, [237, 238] in the case of PROTACs).

As always, new breakthroughs will mostly be dictated by technological developments. For instance, it remains to be seen whether and when cryo-EM will become a routine, high-resolution, and high-throughput tool for

drug discovery. It will also depend on a larger access to the community [239], as has happened with X-ray crystallography at synchrotron facilities. In this regard, it is also essential that a sufficient number of highly trained scientists be formed in both academic and industrial environments. In the same way, SFX holds a huge potential and could become a significant player provided several technical bottlenecks are overcome, but many more facilities are needed to provide enough beam time to users. Maybe the very compact XFEL in construction at the University of Arizona (ASU Compact XFEL) will help address this issue.

We are entering the Big Data era. Structural biology is shifting from 3D to 4D (space + time), i.e. from static to dynamic. Huge progress in our understanding of biology is expected from a combination of 4D structural data with "omics" (genomics, proteomics, metabolomics, etc.), thermodynamics and kinetics studies, molecular dynamics, systems biology, etc. No doubt that one day all this progress will enhance drug discovery and surely structural biology will still have a crucial part to play in this fascinating evolution.

Structural biology will be instrumental in the fight against mutations that cause drug resistance in cancer cells, for instance, in the case of kinases ([240–242] and Chapter 16), and in bacterial [243] and viral (Chapter 22) pathogens, and also in precision medicine [244] by helping understand how natural mutations in human targets alter drug response (see, for instance, [245] in the case of GPCRs). It will also be instrumental in the study of other challenging targets such as proteins from pathogenic organisms causing neglected diseases such as *Mycobacterium tuberculosis* (Chapter 23), enzymes that metabolize drugs such as P450s (Chapter 24), IDPs (Chapter 25), and exciting new targets such as RNAs [246].

Drug discovery is such a complex and difficult endeavor, involving so many different aspects, disciplines, and skills that it is a wonder that quite a number of efficient drugs eventually reach the market to treat various conditions. Structural biology is just another piece of the puzzle, a very powerful one, but unable on its own to guide the medicinal chemist to the optimal drug compound. This is a very exciting period for this fascinating part of the drug discovery toolbox, and probably much more lies in the future.

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