Towards Understanding Drugs on the Molecular Level to Design Drugs of Desired Profiles

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1. Introduction

The process of drug development proceeds through several stages including discovery, product characterisation, formulation, delivery and packaging development, pharmacokinetics and drug disposition, preclinical toxicology testing and IND (Investigational New Drug) application; bioanalytical testing and clinical trials. In general this process is time-consuming and expensive. According to statistical data, from among 50 thousand to 5 million of compounds subjected to screening only 6 become prospective drugs and the whole process from discovery to implementation takes from 12 to 24 years and costs even up to 800 million USD [1]. The number of new approved drugs partially reflects the progress in research and development. Despite enormous financial outlays, there has been a steady decrease in the number of drugs introduced each year into therapy. While in the 1960s about 70-100 new drugs was introduced in the market, in the 1970s, 60-70, in the 1980s 50, in the 1990s 40 while in 2000th only 20. On the one hand, this is a result of increased demands on safety (average number of clinical trials per new drug increased from 30 in the 1970s through 40 in the 1980s, to 70 in the 1990s), which prolonged the development process from 8 years in the 1960s, 12 in 1970s, 14 in 1980s, 15 in 1990s and 21 currently. On the other hand, the so-called "easy" to discover drugs have already been discovered. Currently 75% of expenditures is absorbed by the research ended in failure and as much as 90% of candidates for drug never reach the market.

Therefore, much effort is directed towards development of new theoretical and experimental tools that would increase the effectiveness of the above process, in particular in its first stages leading to a decision whether a given compound is a prospective drug or not. The necessary and reliable data needed to take such a decision are provided first of all by the methods of analytical characterisation of compounds (identification, physical and chemical properties, structure), methods permitting optimisation of the leading structure (structure-activity relation) and pre-clinical study (toxicity). These early stages of pharmacological studies sometimes (but not always) reveal the whole mechanism of action of a future drug.

Recent research work towards drug development has been conducted in two independent directions. On the one hand, completely new drugs or pro-drugs (drug precursors that become drugs only as a result of metabolic transformations *in vivo*) are searched for, while on the other hand, drugs of second and third generation are proposed that are the improved

versions of the so-called first generation ones (e.g. more selective, less toxic, etc.). The search for new areas of applications of the already known drugs is conducted independently. It should be realised that paradoxically the physico-chemical properties or the mechanism of activity of many even commonly used drugs are often unknown. In any case, whenever a completely new or well-known compound becomes a candidate promising to be therapeutically relevant, it must be characterised by determination of its structure, stability, solubility, preferred conditions for maintaining function, toxicity, bioactivity and bioavailability. The physico-chemical methods supported by computer modelling at a high level of theory should always be the first step in drug characterisation.

It is well known that magnetic resonance techniques: Nuclear magnetic resonance (NMR) discovered by Bloch et al. [2] and Purcell et al. [3] in 1946, and Nuclear quadrupole resonance (NQR) originated by Dehmelt and Krüger in 1949 [4] for many years have played invaluable role in the process of drug identification, especially when two or more alternative products could be synthesized or when geometrical isomers should be differentiated. Despite their usefulness, the potential of these methods in drug development at least at first sight seems rather limited in comparison to High-throughput screening (HTS), X-ray diffraction (XRD) or Fourier transform infrared spectroscopy (FTIR). Advances of technology over the last decade have brought modern spectroscopic techniques that are sensitive tools for such purposes, especially in combination with high quality, fast computational quantum chemistry methods like Density Functional Theory (DFT). Apart from revealing structural details (geometrical isomers, enantiomers), the standard key areas for physico-chemical NMR/NQR studies are thermal stability of drugs and structural phase transitions.

A unique and quite a new area of their use is the study of spatial packing. Most of drugs are used in the crystalline form in which the arrangement of molecules forced by weak interactions determines their physico-chemical properties (from melting point, solubility to polymorphism) and thus affects the performance of the drug. What is more, weak, noncovalent and reversible molecular interactions such as hydrogen bondings, *van der Waals* forces, π-π interactions or metal coordination have been only recently found to be crucial for understanding biological activity of drugs and biological processes. As in the absence of conformational effects the environmental effect of the crystal influences the electron density of a single molecule in a very similar way as the enzyme of a drug molecule in the complex drug-enzyme [5], the knowledge of crystal packing provides guidance on the site or method of binding. Thus the problems related to understanding the mechanism of formation and interplay of weak interactions in crystals have evoked recently enormous interest. Additional factor prompting the interest in investigation drugs on molecular level are recent advances in genomics and proteomics that have permitted understanding of internal mechanisms of human diseases. The latter cover the knowledge of the genes alteration caused by disease, its influence on the proteins encoded by them, the interaction of these proteins with each other in living cells, the resulting changes in the specific tissues and finally the affect on the entire body. The achievements in this field delimit new directions in drug discovery and development of drugs addressing the needs of individual patient. Thus new drugs in the near future will be discovered and developed exclusively on the basis of a combination of two factors - the understanding of the mechanisms of diseases and mechanisms of drugs, both at the molecular level.

This chapter highlights the importance of combination of experimental and theoretical methods in investigation aimed at understanding drugs on the molecular level which is fundamental for development of drugs of desirable profiles.

2. Directions in drug design

Throughout the history of medicine the process of new drug discovery has been based on natural sources (berries, herbs, leaves, roots, animal parts or minerals) and drugs have been discovered by **s**erendipity (sheer luck) or in a trial-and-error process [6]. While until the mid-1980s new drugs were discovered mainly by chance, over the next decade, till mid-1990s, the knowledge of structure was the basis for research, then the starting point was to identify a target and a relationship between structure and function. At the beginning of the 21st century a future direction in drug design has been indicated by the knowledge of the human genome, which contains about 23 thousand genes encoding proteins. Although there was some success in treating melanoma, HIV or Parkinson disease, the methods involving gene therapy, whose aims are personalization of drugs, correction of genetic defects or replacement of defective genes are still only a promising methods.

In the last century the demand for effective drugs has increased and a rational drug design has begun to replace old methods. With the progress in the field of chemistry, biology, biochemistry, pharmacology, physics and increase in computational power, drug discovery has become an interdisciplinary area and entered a new phase called Computer-Aided Drug Design (CADD) or Computer-Assisted Molecular Design (CAMD) [7]. The modern methods of rational drug design are capable of designing a biologically active compound, drug (ligand) directly interacting with the distinct so-called molecular target (enzyme, transporter, ion channel or receptor) or components of a microorganism (bacterium, virus, parasite).

In general, CCAD method allows the search for ligands which are predicted to interact strongly with a target or alternatively - the search for a target that will interact strongly with a given ligand. In fact molecules of drugs interact in a very specific way with the targets in organisms and the result of this interaction may include beneficial or adverse effects as well as the excitation of both or biotransformation of the drug [8]. Thus the effective drug design is even more complicated as there are three kinds of targets important in drug discovery process: therapeutic/biological targets (those which drugs specifically bind to and those which elicit therapeutic effects), ADME (absorption, distribution, metabolism, and excretion) associated proteins (responsible for metabolism, important for the efficacy and bioactivity study) and adverse drug reaction (ADR)/toxicity targets (major cause for the failure of drugs).

The CADD approaches can be divided into three categories:

1. **the ligand perspective** - the structure of a ligand or the structure-activity relationship (SAR) are known for a selected ligand and a series of pharmacophores (it requires the assumption that both the protein and ligand have limited degrees of flexibility), which allows identification of the target [9,10]

2. **the target perspective** - the 3D structure of the target is known, which allows identification of the amino acid sequences and their conformations responsible for the binding with ligand [11,12].

3. **the ligand-target interaction perspective** - the interaction between drug and target is known from XDR and NMR structural data [13,14].

The most popular CADD techniques are receptor-based and ligand-based approaches, the former is often called direct while the latter - indirect. Receptor-based drug design requires the knowledge of a 3D structure of the biological target and identification of the protein-binding site or supramolecular host, but the ligand is unknown. Ligand-based drug design

uses a known set of ligands with the receptor site unknown. Both approaches are based on the lock and key fit of receptor and drug and finally use docking, thus are actually very similar. In the receptor-based approach to drug design the structure of a target or a binding site is known from 3D XRD or NMR studies, while a ligand fitting the binding pocket of the target is searched for in the database, which permits elimination of the need for synthesis of new lead compounds. Alternatively the ligand is constructed in such a way as to fit the binding pocket (*de novo design*). The most spectacular achievements of this kind of approach was design of the HIV RT inhibitors based on the known HIV reverse transcriptase structure [15], development of dorzolamide - a carbonic anhydrase inhibitor, an anti-glaucoma agent by Merck [16] or investigation of mechanism of activation of temozolomide and its inhibitor-target interaction with DNA [17]. If a 3D structure it is not available, the homology model of the target can be created on the basis of its amino acid sequence and known structure of proteins resembling the target [18,19]. The method makes use of the fact that sequences of related proteins are similar and thus the protein structures of naturally occurring homologous proteins are also similar

Indirect drug design is based exclusively on the knowledge of the way different ligands bind to an unknown biological target. The structural (in fact stereochemical) and physicochemical features of a set of different ligands (active and inactive) allow the creation of a hypothetical receptor site - 3D-pharmacophore - determining the minimum set of requirements that must be met for the ligand to bind to the target. In fact these requirements describe a few specific interactions responsible for the ligand-target binding i.e. define a simplified model of the receptor site. The 3D-pharmacophore used in conformationally flexible search for ligands provides the best lock-key arrangement with a hypothetical receptor site. There are many different methods of construction of a 3D-pharmacophore, however the main types are: quantitative structure-activity relationship (QSAR), quantitative structure-property relationships (QSPR), molecular shape analysis (MSA), receptor surface models (RSM), comparative molecular field analysis (CoMFA), comparative molecular similarity index analysis (CoMSIA), pharmacophore mapping (PM) etc. The most commonly used are QSAR-based techniques, in which a correlation between the structure and biological activity or physico-chemical properties of a ligand is derived.

In analogy to the direct and indirect approaches in CADD, QSAR techniques can be divided to receptor-dependent (RD) and receptor-independent (RI) ones [20].

In RD-QSAR, models are derived directly from the 3D structures of the multiple ligand-receptor complex. This group of five methods includes: static ligand representation (3D), multiple ligand representation (4D), ligand-based virtual or pseudo receptor models (5D), multiple solvation scenarios (6D) and real receptor or target-based receptor model data (7D) [21]. Using the structure of the ligand-receptor complex in which both ligand and receptor are flexible the induced-fit process is performed using molecular dynamics (MD) simulation. The descriptors are the energies of the interaction responsible for the binding between the analogue molecules and the receptor [22]. An example of a successful application of the RD-4D-QSAR approach is the study of 4-hydroxy-5,6-dihydropyrone inhibitors of HIV-1 protease [23], which permitted prediction of the "bioactive" conformations of the docked analogues into the active site of HIV-1 protease close to those found by XRD. Moreover, the requirements of specific interactions (hydrophobic, steric and hydrogen bonding) between the enzyme active site and the binding site of analogues of 4-hydroxy-5,6-dihydropyrone inhibitor with HIV-1 protease, indicate directions of development of new lead HIV-1 protease inhibitors [23].

In RI-QSAR, the geometry of the receptor is not available, or it is neglected because it is not credible or its binding with ligand is not recognised. This group of methods include the "classical" (zero-dimensional), one-dimensional (1D), two-dimensional (2D), three-dimensional (3D), and four dimensional (4D) QSAR approaches [24]. The calculated descriptors are atom and molecular counts, molecular weight, sum of atomic properties (0D-QSAR); fragment counts (1D-QSAR); topological descriptors (2D-QSAR); geometrical, atomic coordinates, or energy grid descriptors (3D-QSAR); and a combination of atomic coordinates and sampling of conformations (RI-4D-QSAR) [24]. Commercial drugs developed with the aid of classical QSAR include: Norfloxacin (broad-spectrum antibacterial agent) Kyorin 1983; Metconazole (Wheat Fungicide), Kureha 1994; Lomerizine (Antimigrane, Antiglaucoma) Organon Japan-Upjohn 1999; Flobufen (Long-acting Antiinflammatory) Kuchar et al., Virbac 2000 [25]. 3D QSAR approaches CoMFA [26] and its extension CoMSIA [26] are popular thanks to their ability to generate highly predictive and easy interpretable models even for flexible molecules, despite the fact that only one conformation of each compound is considered. The CoMFA approach uses in its standard implementation only Lennard-Jones and Coulomb potentials, while in the extended version also the hydrophobic and hydrogen-bond fields are taken into account [27]. The field type used in CoMSIA is similar. Instead of field descriptors based on potentials, the use is made of the so-called similarity index describing steric, electrostatic, and hydrophobic similarity or dissimilarity of molecules. Additionally in CoMSIA, Gaussian-type functionals eliminating singularities at the atomic positions are applied [28-30]. Successful application of CoMFA is illustrated by the studies of inhibitors: trypsin, thrombin, factor Xa [30] or thermolysin [31]. Currently, one of the most advanced QSAR-related techniques is the 4D-QSAR approach [32] which eliminates the restriction to a single conformation and allows averaging of the conventional 3D-QSAR descriptors thanks to the assumed conformational flexibility. It allows consideration of the effects of multiple conformations, alignments, and substructure groups i.e. extension of 'QSAR degrees of freedom' in comparison to those used in 3D-QSAR analysis. In this kind of approach, the so-called grid cell occupancy descriptors are used i.e. the occupancy frequencies of the different atom types (any type, nonpolar, polar-positive charge, polar-negative charge, hydrogen bond acceptor, hydrogen bond donor and aromatic) in the cubic grid cells [33,34]. An example of successful application of the RI-4D-QSAR approach is the study of 5'-arylthiourea thymidine analogues, showing inhibitory activity against thymidine monophosphate kinase from *M. tuberculsosis* (TMPKmt) [35]. Recently, different RI- and RD-4D-QSAR approaches were successfully applied to a variety of enzyme inhibitors of different drug targets, such as HIV-1 protease [36,37], HIV-1 integrase [38], p38-mitogen-activated protein kinase (p38-MAPK) [39], 14-α-lanosterol demethylase (CYP51) [40], enoyl-ACP reductase from *M. tuberculosis* (InhA) [41] etc.

Construction of a model requires determination of the structural elements of the target that would be necessary for the ligand to get recognised and to form a stable active complex. Depending on the availability of experimental data, one or a combination of the above mentioned methods is used. The optimum situation for CADD application is when both a group of ligands of defined activity and the spatial structure of the target are known. The search stage called molecular docking in which a ligand is matched to the receptor site and the quality of the fit (most often expressed in energy) is evaluated, is actually very similar irrespective of whether a direct or indirect approach is used. In both cases it is based on

algorithms which simulate the docking process of ligands to the target [42]. The receptor binding pocket or its homology model can be used with auto-docking to find the most appropriate ligands or alternatively 3D-pharmacophore can be used in conformationally flexible (steric interactions) search for ligands that fit to the spatial distribution of the receptor. In order for the ligand-target interaction to occur, a few conditions must be met, such as a sufficient concentration of the drug which depends on the quantity of drug administered, its capacity to reach the target and its affinity and specificity for the target. Only specificity and affinity can be modelled, because they are directly related to the structural and electronic complementarities of the drug and target. The structural complementarity is strictly mechanistic and can be described as hollow/bump or key/lock adjustment. It is facilitated by the flexibility of drug and target i.e. their susceptibility to conformational changes which ensure the mutual compatibility of these two molecules. The electronic complementarity can be described as attraction/repulsion depending on the mutual relation between electron densities of the drug and target. In general it facilitates the approach of the drug to its target and in a consequence its binding. The most desirable combination is both a structural complementarity and an electronic complementarity between drug and target, as it ensures the best affinity. The interaction established between drug and target stabilizing the complex is rarely covalent and typically of low energy (electrostatic, van der Waals, hydrogen bonding). Therefore docking is accomplished by either geometric matching of the ligand and its receptor (faster) or by minimising the energy of interaction (slower, more accurate). Geometric matching is often replaced by the matching based on charge distribution, bond vectors, conformational flexibility, hydrophobic properties or molecular fragments.

Thus the knowledge of the 3D structure and/or the physico-chemical properties (electronic, steric, hydrophobic features etc.) of the drug and its biological target as well as the knowledge of their fluctuations related to their molecular motions, polymorphic transitions or shifts in tautomeric equilibrium of drug, is fundamental to understanding molecular recognition and intermolecular interactions participating in drug-target binding.

Many experimental and theoretical, very sophisticated techniques, provide complementary types of data, which after quick selection are considered to be required or not for the full description or prediction of drug - target interaction. With the development of experimental (e.g. XRD, NMR, NQR) and theoretical methods (e.g. ab initio, DFT at different levels) not only the number of recognised 3D structures of ligands and targets has dramatically increased, but also the amount of information on structural dynamics and electronic properties of drugs. The increased availability of these data is crucial for rational drug design, which in general has to involve many different pathways to achieve one common goal that is a drug more efficient and selective to its therapeutic target which exhibits efficacy in vivo and shows low toxicity. Achievement of this goal does not seem possible without understanding drugs on the molecular level.

3. Understanding of drugs on the molecular level

From the chemical point of view, the most important components of drugs i.e. Active Pharmaceutical Ingredients (API), a substance of a drug which is biologically active, and excipients, inactive substances used as a carrier for the API, are composed of small, organic, low-weight molecules (molecular mass below 100 Da, only rarely 500 Da) mainly obtained through chemical synthesis. However, the interest in drugs available through biological

processes (biopolymer-based) steadily increases. It is difficult even to estimate the total number of currently known drugs and targets, as it is continuously in change, the data are scattered over multiple resources and available in a non-uniform manner. Comprehensive Medicinal Chemistry (CMC) lists 8,659 [43], PharmaPendium covers over 4,000 [44], Thomson REUTERS Forecast [45] tracks approximately 3,500 in clinical-stage and marketed drugs (700 strategic drugs). One of the largest databases – the Therapeutic Target Database [46] contains information about 11,978 drugs (including 1,514 approved, 1,282 in clinical trial, 9,182 experimental drugs and 3,645 multi-target agents) covering 140 therapeutic classes and 1,973 targets (including 358 successful, 254 clinical trial, 44 discontinued and 1,317 research targets) covering 61 protein biochemical class. Another database, BindingDB, contains 316,172 small drug-like molecules and 721,721 measured binding affinities for 6,179 protein considered to be drug-targets [47]. The DrugBank database [48] – a unique resource that combines chemical, pharmacological and pharmaceutical data with drug target (i.e. sequence, structure, and pathway) contains 6,816 drug entries including 1,437 FDA-approved small molecule drugs, 134 FDA-approved biotech (protein/peptide) drugs, 83 nutraceuticals and 5,194 experimental drugs.

According to World Health Organization (WHO) reports [49] the number of essential drugs has nearly doubled, from 186 in 1977 (1st edition) to 364 in 2011 (17th edition). It should be remembered that the list of essential drugs is extremely selective and includes only "*those drugs that satisfy the health care needs of the majority of the population; they should therefore be available at all times in adequate amounts and in appropriate dosage forms, at a price the community can afford*" [50]. The US Food and Drug Administration (FDA) agency lists about 800 approved API and there are over 100,000 drug products created from those API collected in Green Book, while 14,309 (primarily small-molecule drugs) are collected in US FDA's Orange Book [51] and about 6,000 biological drugs are listed by the Centre for Biologics Evaluation and Research (CBER) [52]. However, when duplicate APIs, salt forms, supplements, vitamins, imaging agents etc. are removed, the list of unique drugs can be shortened to 1,357, of which 1,204 are 'small-molecule drugs' and 166 are 'biological' drugs. FDA list contains also 11,066 inactive ingredients i.e. excipients.

The widely used classification system for API substances is the Anatomical Therapeutic Chemical (ATC) system [53] introduced in 1976 and controlled by Collaborating Centre for Drug Statistics Methodology (WHOCC). ATC categorizes API at different levels: anatomy, therapeutic properties and chemical properties according to therapeutic aspects. The modern, very recently introduced, system of classification [54] assumes that all current drugs with a known mode-of-action act through 324 distinct molecular drug targets and classifies them according to known targets (biochemical structures like: enzymes, substrates, metabolites and proteins, receptors, ion channels, transport proteins, DNA/RNA and the ribosome, targets of monoclonal antibodies; various physicochemical mechanisms and unknown mechanism of action). Current therapy is based on the above mentioned molecular targets [55,56] of which 270 are encoded by the human genome (45% - G-protein coupled receptors, 28% - enzymes, 11% - hormones, 5% - ion channels and 2% - nuclear receptors). Because each of 10 genes which contribute to multifactorial diseases are typically linked to other 5 - 10 gene products in physiological circuits suitable for pharmaceutical intervention, it is expected that in fact there are at least 10-20 times more targets [57]. Their identification is a priority as it determines the discovery of innovative drugs**.**

Many API's are intended to be used in solid dosage formulations, which is generally preferred for oral route of administration due to greater stability, smaller volume, accurate

and flexible dosage and easy manufacturing. In fact oral small-molecule API's target as many as 227 molecular targets [56]. The modern system of classification of APIs according to known targets in fact classifies them according to their mechanisms of action i.e. the API effect at the molecular level. Understanding the API effect at the molecular level requires the knowledge of fundamental data including spatial 3D structure and physico-chemical properties of API. A combination of the experimental (XRD, NMR, NQR spectroscopic techniques) and theoretical data (ab initio, DFT calculations) in a synergistic manner not only provides the data on structure or properties but also allows prediction of the way in which the specific molecular changes will alter the features of API. This, in turn, indicates the directions of design of new API by taking into account such factors as predisposition to participate in certain intermolecular and intramolecular interactions, tendency to polymorphic or tautomeric transitions, required stability (thermal or photo or radiation stability) or solubility. All these factors are closely related to biological effect therapeutic or toxic thus provide the basis for understanding drugs at a more fundamental level. The examples discussed in the next section will cover some, but not all these aspects.

3.1 Spatial 3D structure

In 1868 Brown and Fraser [58] discovered that biological activity of compounds is related to their chemical structure. Until the mid 1990s, the 3D structure was in fact the main a basis of drug design, but even nowadays it is still a key factor. As previously mentioned, the knowledge of the spatial 3D structure of API itself or its biological target is the core of CADD techniques. 3D structure of API or target can be determined either experimentally, using direct or indirect methods, or can be predicted theoretically.

3.1.1 Experimental structure

XRD

The most important sources of information on the drug structures are direct crystallographic methods, i.e. the methods which derive diffraction phases directly from experimentally measured diffraction amplitudes. The first atomic-resolution crystal structure solved by Bragg in 1914 [59] was that of NaCl - a substance playing a crucial role in maintaining the fluid and electrolyte balance. The standard single crystal XRD, actually providing a resolution of 0.5-3.0 Å, is a main source of information about the molecular conformation and packing in the crystalline structure. For small molecules, the crystal structures are usually well resolved. However, high quality single crystals of sufficient size of many pharmaceuticals have proved impossible to grow, while polycrystalline powders can be readily made. For such substances, the powder X-ray diffraction (PXRD) or small-angle X-ray scattering (SAXS) are used to obtain the relevant structural information, which is in fact less detailed. Despite this, PXRD pattern, considered as a characteristic fingerprint of a crystalline phase, is used to identify and characterise crystal forms including polymorphs (1800 of such a patterns are stored at CSD).

A significant disadvantage of XRD from the point of view of APIs studies is not only the necessity of obtaining crystals, but the difficulty in determining the positions of light atoms such hydrogen (only one electron) resulting in imprecise description of weak intermolecular interactions. Although, various parameters describing the covalent geometry (i.e., bond lengths, bond angles, planes, and chirality) are determined to a very high accuracy [60], the degree of uncertainty of the parameters characterising the weak, nonbonding interactions is

still high. The application of neutron diffraction (ND), which due to the interaction of radiation with atomic nuclei gives highly accurate positions of tiny atoms (hydrogen), partially solves this problem. The widely used approach for this purpose is the Hansen–Coppens formalism [61] in which individual atomic densities are described in terms of a spherical core and valence densities. Expansion of the atom-centred spherical-harmonic functions is the main idea of this so-called multipole approach. Another solution is modelling with the use of independent atoms [62], or partial optimisation using quantum chemistry methods ab initio or DFT [63]. However, irrespective of the method, the final result becomes in fact not purely experimental. The most accurate API structures, with respect to both molecular geometry and motional description, still come from low-temperature ND [64]. Despite the above mentioned inconveniences, XRD is the starting point for gathering information about APIs.

XRD is also the oldest and major source of 3D structures of targets as well as drug-target complexes [65,66]. In 1965 Johnson and Philips [65] solved the structure of lysozyme (with 2 Å resolution), a 14,7 kDa enzyme which can be found in tears, saliva and egg white. A milestone in the development of CADD was the invention of X-ray structural analysis techniques that would allow examination of structures of very large molecules - proteins, enzymes. The largest known and resolved by XRD protein is human Titin, which consists of 34,350 amino acids, with the molecular weight of the 'canonical' isoform of the protein being approximately 3,816,188.13 Da [67]. However, its use is still marred with a number of restrictions, the method can be applied to good crystals, so its use is restricted to solids (difficult to obtain for targets), it gives no possibility to examine small parts in the large molecule in details, it is unable to reveal many conformations (molecular motions are neglected) or to determine the secondary structures, which is crucial for the target or drug-target complex. A common but rarely mentioned problem of X-ray crystallography and electron crystallography is radiation damage of the samples studied.

The other worth mentioning techniques, complementary to XRD, are the Small-angle X-ray scattering (SAXS) [68], atomic forces microscopy (ATF) [69] or cryo-electron microscopy [70], which allow studies under physiological conditions and are used for studies of micro or nanoscale particles. However, actually their resolution is still lower than that of XRD. One more technique complementary to XRD is the high-resolution electron microscopy (HREM), which can be successfully applied for crystal structure analysis when the grain size is too small or periodicity is imperfect. Its main advantage over XRD is more convenient observation of light atoms in the presence of much heavier atoms. HREM is able to produce a microscopic image and a corresponding diffraction pattern in the atomic scale, however, for crystalline samples the resolution is too low to reveal individual atoms [70].

There are many other techniques that are useful in the study of crystals, but their description is outside the scope of this paper.

NMR

Some of the limitations of XRD can be easily overcome by other techniques like NMR, which reveals a 3D structure of a molecule not only in crystal but also in its natural - biological environment. Solid state NMR (SS NMR) is one of the most important tools for structural investigation of solid APIs for which it delivers data complementary to XRD (precise bond lengths and angles within a molecule, intermolecular bond lengths and angles). It can be said that it adds the time-dependence to the XRD parameter-set, which allows studies of the kinetics of reactions, molecular motions, conformational changes etc. Depending on the type

of experiment, different NMR parameters: isotropic chemical shifts, coupling constants, rate constants, chemical shift tensors, spin diffusion and dipolar interactions etc., provide different information about the chemical environment of the selected nuclei in a molecule. In general, the principles are the same for solution and SS NMR. However, low sensitivity and severe line broadening originating from chemical shift anisotropy (CSA) and dipolar interactions are serious inconveniences for SS NMR. Three techniques: cross polarisation (CP), magic angle spinning (MAS) and high-power[1]H decoupling are applied to overcome the problems encountered in the solid state NMR. For small compounds like APIs each chemically inequivalent site and functional group can be distinguished on the basis of chemical shift. In fact, unique SS NMR spectroscopy capabilities allow differentiation of isomorphic solids, which are difficult to distinguish by XRD. When the spectra are complicated, the spin-spin splittings or *J*-couplings are used to elucidate and verify a possible structure but this can prove insufficient [71]. Moreover, in large molecules such as e.g. targets, the number of chemical shifts can be extremely large (even several thousand) and can suffer from incidental overlaps. The use of sophisticated techniques like multidimentional 2D-4D NMR is then required [72-76]. The most popular are 2D homonuclear NMR experiments: correlation spectroscopy (COSY) [71] and total correlation spectroscopy (TOCSY) [72], which correlate different spins via scalar spin–spin couplings or nuclear Overhauser effect spectroscopy (NOESY) [73], which describes dipole–dipole coupling between pairs of nuclei or Incredible Natural Abundance Double Quantum Transfer Experiment (INADEQUATE) [77], which correlates different carbon nuclei. Each of them produces two-dimensional spectra mapping chemical shifts and reflecting adjacent atoms (COSY) or all atoms if they are connected by a continuous chain of protons (e.g. side chains of amino acids) (TOCSY). Apart from this, the method is also able to reveal the spatial proximity between the atoms (NOESY). As the intensity of a NOESY peak is reciprocally proportional to the 6th power distance, thus it reveals the distance. Because the intensity-distance relationship is approximate thus NOESY derived distance is not exact. The other disadvantages of NOESY are separations no farther than 5 Å and the ambiguous assignment of peaks that are close in space but do not belong to the sequential residues. For such a purpose the technique of labelling of proteins (with ^{13}C, ^{15}N or ^{2}D) is used. Instead of NOESY one or more of the six HSQC-type experiments (HNCO, HNCACO, HNCA, HNCOCA, HNCACB and CBCACONH) can be used, which are capable to resolve overlap in the carbon dimension. The most widely used is the HCCH-TOCSY method (i.e. TOCSY resolved in an additional carbon dimension). Apart from distances, NMR provides estimation of the torsion angles (on the basis of *J*-coupling constants - Karplus equation or chemical shifts). The introduction of isotope labelling and multidimentional experiments extended the area of NMR applications to targets. However, fast relaxation and broader and weaker peaks, which can easily disappear are important limitations. Recently, two techniques dedicated to large target studies have been introduced: residual dipolar couplings (RDC) [78,79], which can be used in particular for the reveal the orientation of the N-H as well as the whole structure refinement, transverse relaxation optimised spectroscopy (TROSY) [80,81] and cross-correlated relaxation-enhanced polarisation transfer (CRINEPT) [82]. These modern techniques allow studies of targets larger than 150kDa. It should be emphasised that the structural information provided by NMR is indirect because no simple relationship between the experimental data and Cartesian coordinates is available. While XRD delivers the approximated coordinates of atoms in target, the NMR gives the estimations of distances between the pairs of target atoms. Therefore, the final conformation

of a target is obtained, by solving a geometry-distance problem. Because the actual conformation depends on the properties of the environment, thus the use of NMR to study its influence is possible, for example the influence of different solvents. The resolution of NMR, 2-5 Å, is much lower than XRD since the information got from the same material is much more complex. For the same reason, NMR allows determination of the 3D structure of smaller molecules than XRD. The most spectacular NMR result is the determination of the protein in complex with the 900 kDa chaperone GroES-GroEL [83]. Unique advantages of NMR are the capability of solving secondary structures [84-86] and mapping the binding sites of the substrate [87,88]. At the current state-of-the art methodologies, XDR is still much more efficient than NMR, even in solving a structure of a target. While NMR is expensive - requires isotope labelling, sophisticated spectrometers and time consuming - a determination a high-resolution structure of proteins <40 kDa may take even a year, XDR routinely solves it in weeks to months. Despite these limitations, the role of NMR is continually expanding, as evidenced by the fact that NMR has evolved into one of the two major techniques for elucidation of structure and dynamics of targets and target-drug complexes.

Taking into account advantages and disadvantages of both methods the optimum method for solving structure of API is XRD, while a combination of XRD and NMR studies provides 3D structures of targets or drug-target complexes.

Against the background of the above mentioned methods, NQR, which gives quadrupole coupling constants (e^2qQ/h) and asymmetry parameters (η) from experimental frequencies, seems to have very limited capabilities. Moreover its use involves a number of restrictions; it can be applied only to high purity solids (crystalline or polycrystalline), only for selected isotopes and practically its applications are limited to API. In fact, however, this underappreciated technique has some unique advantages and is an effective tool for preliminary study of crystal structure in the absence of X-Ray data. It allows measurements of distances but only NH or OH bond lengths, gives the number of inequivalent molecules in the elementary cell, permits differentiation of isomers, enantiomers, polymorphs etc.

Successful application of NQR to analysis of structural aspects is illustrated by the studies of the most widely used cytotoxic agent most effective in the treatment of epithelial malignancies such as lung, head and neck, ovarian, bladder and testicular cancer, i.e.cisplatin cis -diamminedichloroplatinum (II). Although cisplatinum and its complexes are widely used as anti-cancer drugs, neither its isomer – transplatin *trans* - diamminedichloroplatinum (I) – nor its complexes have therapeutic properties. Possible explanations of different biological activity of the *cis* and *trans* isomers are that *cis* compounds make platinum-DNA adducts inhibiting DNA replication or transcription to a greater extent than those formed by transplatin, and alternatively, that DNA adducts formed by *trans* compounds may be repaired more efficiently [89]. Thus, differentiation between the *cis* and trans isomers is essential. Isomers distinction for any platinum compounds showing *trans* – *cis* isomerism with ^{35}Cl-NQR is easy as the difference in the resonance frequencies is high enough and reaches 2.13 MHz: 16.18 MHz for *cis*-platinum and 18.31MHz for *trans*-platinum [90]. Another interesting example of NQR capabilities is the study of serine, which occurs in the active sites of many enzymes like chymotrypsin, trypsin and plays an important catalytic function. The enzyme serine racemase converts L-serine into the less common D-isomer (a co-agonist of the N-methyl-D-aspartate receptor in glutamate neurotransmission) and can be used to develop treatments for neurodegenerative diseases (schizophrenia). The sophisticated high sensitivity SQUID technique allows studies

of chirality i.e. distinction of L-serine and D-serine and its racemate DL-serine [91] on the basis of small differences in the frequencies (0.018 and 0.023 MHz), quadrupole coupling constants (0.004 MHz) and asymmetry parameters (0.066 MHz). One more illustrative example are NQR studies of diuretic drugs: the highly active and thus widely used one - hydrochlorothiazide (HCTZ) and the weak one and thus used only to alter the mode of furosemide activity - chlorothiazide (CTZ). Both are used in the treatment of hypertension, congestive heart failure, symptomatic oedema and prevention of kidney stones. The difference is only in one bond (in CTZ either the bond N(2)–C(3) or C(3)–N(4) is double, while in HCTZ one hydrogen atom occurs at N(2) and N(4), and the two bonds N(2)–C(3) and C(3)–N(4) are single). This structural difference results in a significant (1.26 MHz) difference in frequencies, which makes the distinction of both compounds very easy [92].

As far as large structures are concerned, a striking example of NQR application was a direct observation of Zn^{2+} in Carbonic Anhydrase (CA), the first enzyme whose activity was recognised to be related to the presence of Zn^{2+}, which was postulated as the activator of bound H_2O. CA, which commonly occurs in all mammalian tissues, plants, algae, and bacteria, is perhaps one of the best recognised metalloprotein. The ^{67}Zn QCC values, being sensitive to changes in the structure and bonding associated with water or hydroxide have been found independent of pH over the range of 5 to 8.5 [93], whereas, according to the hitherto commonly assumed model, Zn^{2+} should be coordinated by H_2O, and as a result, at these pH values the ^{67}Zn NQR spectrum should be three to five times broader than that at pH 8.5. This observation was in contradiction to the widely accepted mechanism and led to acceptance of an alternative one proposed by Merz, Hoffmann, and Dewar [94]. The above example demonstrates the significance of zinc NQR spectroscopy in delineating the structure and mechanism of activity of this class of metalloproteins.

These examples show that NQR has a potential to provide vital structural information about the drug, target and the drug-target interaction.

3.1.2 Predicted structure

Prediction of the structure of single molecules, the so-called geometry optimisation, is available within different approaches starting from molecular mechanics up to very sophisticated levels of the quantum chemical theory. But a single molecule structure is far from that in crystal, especially for flexible molecules. Although it is known that molecules in crystals are bonded via intermolecular interactions, which are weak and numerous, but they act at long ranges and have little directionality, which makes crystals difficult to model [95]. Thus crystal structure prediction (CSP), which includes the prediction of the crystal structures of solids from first principles, is a much more complicated task. Reliable methods for the prediction of crystal structure only on the basis of molecular structure have been searched for since the 1950s [95]. Until recently, crystal structure could not have been predicted computationally from first principles because of the nature of forces acting between the molecules in crystals. Reliable CSP requires assumption of clearly defined criteria of the quality of structure reproduction. One possibility is a comparison of the predicted structure with those experimentally determined. A good test of CSP quality is the validity of reproducing structures that differ only slightly as those of different polymorphs. Indeed, many unsuccessful attempts to predict the structures of polymorphs of sulphonamide have been undertaken. It was believed that the reason for the failure was the kinetic nature of crystallization. But it turned out that successful CSP for small drug molecules is possible [96,97]. Only recently, the structures of three known polymorphs of

sulphanilamide have been correctly predicted from ab initio [98]. The prediction of target or target-drug structure is still an issue for the future.

Much faster is the CSP validation based on experimental parameters. For example NQR, which is a very sensitive technique, provides parameters directly describing the local environment of a nucleus in the molecule. However CPS, based only on these data is impossible, but in combination with DFT it can be used for example to evaluate the degree of structural refinement. A valuable example is triclosan (5-chloro-2-(2′,4′-dichlorophenoxy)-phenol, TCS) active pharmaceutical ingredient (API) of a known potent wide spectrum of antibacterial, antimicrobial and antifungal agents used in many drugs as well as antiseptic or disinfectant formulations. The crystalline structure of TCS, which crystallizes in the space group P3$_1$ with one molecule in the asymmetric unit has been solved by XRD and refined to a final *R*-factor of 2.81% at room temperature [99] and 3.74% at 90 K [100]. The conformation adopted by diphenyl ether of TCS in solid is temperature independent, typical of diphenyl ethers but the opposite to that adopted when it is bound to different inhibitors. The importance of this finding is the suggestion of the presence of two enantiomers of triclosan, which would nicely explain a wide spectrum of its activity. The smooth changes in NQR frequency with temperature increasing from 77 K to 300 K [101] and similar parameters of the unit cells at RT and at 90 K (a=12.5225 Å, b=12.5225 Å and c=6.6809 Å [100] versus a=12.6410 Å, b=12.6410 Å and c=6.7163 Å [99]) confirmed no phase transformation from RT to 90 K during the data collection. The differences in RT and 90K structures seem relatively small but the NQR spectrum of triclosan was much better reproduced at the B3LYP/6-311++G(d,p) level of theory assuming the room temperature structure than 90K one. The scattering of NQR frequencies was found to be a good indicator of the quality of the crystallographic structure resolution [99].
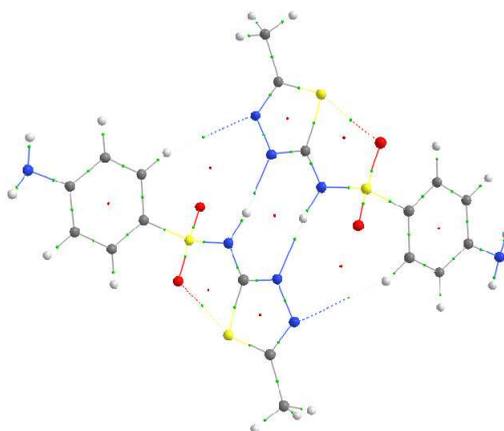

Fig. 1. Predicted structure of sulfamethizole.

NQR combined with DFT can be also used for structure prediction. An interesting example is the structure of sulfamethizole, known to be able to crystallize in different polymorphic forms [102,103] of which none, even the most stable one, was solved. In a single molecule of sulfamethizole there are four nonequivalent nitrogen positions, but within the NQR experimental resolution five nitrogen positions have been detected [104]. This indicated

either two nonequivalent molecules in the unit cell or two polymorphic crystal forms of sulfamethizole. The lines at the–NH- and –NH$_2$ nitrogen positions do not show any splitting within the experimental resolution. A possible explanation of the ^{14}N NQR spectra is that the sulfamethizole molecules form slightly asymmetric hydrogen bonded pairs, similar to the symmetric pairs formed by the acetazolamide molecules. The NQR parameters obtained by B3LYP/6-311++G(d,p) assuming putative dimeric structure, Fig.1., were in a very good agreement with the experimental data [104].

Studies of isostructurality and annular 50:50 prototropic tautomerism of pentabromobenzimidazoles make another example of the successful prediction of crystalline structure performed by NQR and DFT/QTAIM combined [105]. On the basis of three known XRD structures of 4,5,6,7-tetraiodo-1H-benzimidazole, 4,6-dibromo-5,7-diiodo-1H-benzimidazole and 4,6-dichloro-5,7-diiodo-1H-benzimidazole the crystalline structures of a set of pentabromo-benzimidazoles have been proposed and the most stable polymorphic form (alpha) has been predicted.

3.2 Intermolecular interactions pattern

Intermolecular interaction pattern can be analysed using structural data, but more detailed information can be derived from the electron density distribution, which can be determined either experimentally or theoretically. The electron density distribution is a purely physical property of molecules and an observable, thus it can be approached experimentally directly (as maps) or indirectly (as parameters).

For single molecules electron density distribution can be derived from gas electron diffraction (GES) while for solid formulations of drugs from diffraction methods like XRD or neutron diffraction. Using the intensities (measured directly) and phases of the scattered X-Rays (obtained indirectly for example by intelligent guess i.e. Hautpmann-Karl method, through isomorphic substitution or a comparison of intensities at a range of wavelengths near the K_α edge of a heavy atom in the crystal) the electron distribution in the crystal after 3D Fourier transform can be mapped as a contour plot, the so-called Fourier map of electron density. According to Parseval's theorem [106], the rms error in Fourier map is proportional to the rms error in the structure factor thus minimisation of rms error in electron density distributions ensures getting a structural factor that minimises the rms error in the complex plane. Importantly, X-ray is in fact an imaging technique because the Fourier transform of the observed structure yields an electron density map of the molecule.

SS NMR, which is complementary to XRD taking into account structure solving, does not deliver an electron density distribution per se but the variations in chemical shielding (or its isotropic chemical shifts) that reflect the variations in this distribution (lower electron density is reflected by smaller chemical shifts). The reliable and experimental parameters directly describing electron density distribution - quadrupole coupling constants and asymmetry parameters - are delivered by NQR. They reflect the electron distribution in the vicinity of the quadrupolar nucleus, thus provide a very sensitive tool for investigation of short range interactions (in contrast to the long range periodic order seen by the XRD). The disadvantage of NQR is the fact that it is not an imaging technique i.e. it does not deliver Fourier maps.

The electron density distribution can be also obtained theoretically using quantum chemical calculations at different levels of theory (semiempirical, *ab intio* or DFT).

Detailed analysis of topology of the electron density distribution, obtained experimentally or theoretically, can be performed within the Quantum Theory of Atoms in Molecules,

QTAIM formulated by Bader [107]. Within this approach the electron density $\rho(r)$ of a molecule, treated as a scalar field, can be examined by analysis of its gradient vector field and a Laplacian. An atom is defined as a region of real space bounded by surfaces through which there is a zero flux in the gradient vector field of the electron density. The values of $\rho(r)$ at the start and end points of a gradient path, which follows the largest increase in $\rho(r)$, take extremes: maxima or minima, or saddle points in the electron density. Depending on the nature of the extreme they are called the nuclear attractor-, bond-, ring-, and cage-critical points and denoted as NACP, BCP, RCP and CCP, respectively. The type of the critical point can be easily determined with the help of a Hessian matrix composed of nine second-order derivatives of $\rho(r)$. The knowledge of electron density distribution allows evaluation of many one-electron properties (e.g. electric dipole moment, electrostatic potential, electric field, electric field gradient, Laplacian of electron density, molecular orbitals) describing the bonds in molecules and intermolecular interactions pattern in molecular crystals. The electron density $\rho(r)$ at BCP and its Laplacian are the indicators of the character of the bond and allow classification of the bondings according to the following rules:

- $\rho(r) > 0$, $\Delta\rho(r) < 0$; covalent (pure closed-shell)
- $\rho(r) \sim 0$, $\Delta\rho(r) > 0$; ionic (pure shared shell)
- $\rho(r) > 0$, $\Delta\rho(r) > 0$; charge shift (transit closed shell)

A number of books [107,108] and review articles [109-111] have described this technique and given examples of its application. The electron density (and thus properties of a molecule) varies according to the local geometry (binding partners, bond lengths, angles between bonds etc.) or external factors (temperature, pressure, solvent, external electric field etc.). Additionally, the electron density can be also influenced by another molecule to the extent dependent on polarisability. This factor is very important for slightly charged atoms having a great number of electrons, like sulphur or iodine, which permits their participation in intermolecular interactions.

Highlighted below are the examples wherein SS NMR and especially NQR spectroscopy combined with DFT has been used to extract detail information on APIs. In this section, particular instances of application of NQR spectroscopy, which provide unique quality information for CADD i.e. for crystal engineering strategies and structure-property relationships are presented.

3.2.1 Polymorphism

Polymorphism (Greek: *polys -multiple, morfé - shape*) is the ability of a substance to exist in more than one crystalline form (polymorph) depending on the crystallisation conditions. While initially most polymorphs have been discovered by chance [111-115], recently identification of polymorphism is a result of a systematic search or is even required by the FDA standards. According to McCrone suggestion, "*in general, the number of forms known for a given compound is proportional to the time and money spent in research on that compound*" [116]. Approximately 70% of API exhibit polymorphism and rarely the differences between two polymorphs and their properties are subtle (e.g. aspirin - acetylsalycilic acid [117]).

Polymorphs differ in physico-chemical properties (e.g. solubility, dissolution rate, permeability, chemical reactivity, melting point, optical and electrical properties, vapour pressure, crystal shape, compressibility, density, hardness, resistance to degradation), which makes a very important problem from the point of view of pharmaceutical industry. It can

cause crucial problems not only with the manufacturing or the quality of the formulation but also with the biodisponibility of the active substance and drug stability (i.e. shelf life of a drug). Thus characterisation of solid-state properties of API as well as excipients is of particular interest. In fact API exhibiting structural polymorphism received regulatory approval for only one single crystal form or a specific polymorph. An example is Cefdinir, broad-spectrum antibiotic, for which a lot of companies (Fujisawa, Biochemie, ACS Dobfar, Orchid, Abbott, Aurobindo, Novartis, Lupin, Rambaxy) patented 11 polymorphs related to 5 crystalline forms [118], atorvastatin calcium - a statin lowering blood cholesterol, for which more than 60 solid forms have been patented [119], piroxicam - a non-steroidal anti-inflammatory drug, synthesized in more than 50 forms [120] and sulfathiazole a local antimicrobial agent for which more than 100 forms have been described [121]. From among multicomponents the one showing the optimum properties from the biological activity point of view is chosen.

For pharmaceutical companies, the phenomenon of polymorphism is rather a disadvantage, however sometimes it is used to extend original patents on existing API ,but this procedure requires checking that one polymorph does not decompose or transform to another (infringement of another patent). A well-known example is the patent litigation between pharmaceutical companies Glaxo and Novopharm concerning two polymorphs of ranitidine hydrochloride – a histamine H_2-receptor antagonist that inhibits stomach acid production [122], the problem of "disappearing polymorphs" of ritonavir [123] – an inhibitor of HIV-protease manufactured by Abbot Laboratories or paxil - anti-depressant manufactured by SmithKline Beecham (GlaxoSmithKline). Therefore, the original companies use a stable polymorph, while the generic companies a meta-stable polymorph. The way of labelling of polymorphs is inconsistent (e.g. I, II, III …; A, B, C…; α,β,γ...) and has no relation to a polymorph stability; it occasionally happens that identical forms are named differently.

In general, polymorphism results from different crystal packing of rigid molecules (packing polymorphism) e.g. acetazolamide, cloxiquine, the presence of different conformations of a flexible molecule (conformational polymorphism) e.g. sulfanilamide, chlorpropamide, or can be a result of inclusion of water or solvent molecules in the lattice i.e. hydration or solvation, respectively (pseudopolymorphism or solvatomorphism) e.g. chloral hydrate, cefdinir. The packing polymorphism is much rarer than the conformational one. In practice usually the mixed types of polymorphism are encountered. The most frequent case is dimorphism. There is a tight connection between the synthons arrangement (packing polymorphism) or the molecular conformation (conformational polymorphism) and the crystal packing in a an elementary unit. The weak noncovalent interactions (hydrogen-bonds, Van der Waals forces, coulombic interactions, steric repulsions π-π interactions) are responsible for the bonds between molecules or supramolecular synthons in the crystal. In general, the molecular energy and the lattice energy of the crystals are not independent, but in fact they control each other. Changes in the weak interaction pattern are able to induce further changes for example in torsion angles, which can result in stabilization of different molecular conformations close to the most stable equilibrium one. Consequently, various nearly energetically equal molecular conformations can force crystallization in different polymorphs. Therefore, a study of molecular motions is sometimes used for the characterisation of polymorphs. Elucidation of the very origin of polymorphism is often crucial to understanding drugs at the molecular level. Polymorphism is a widespread phenomenon among small molecules of APIs, mostly because of their flexibility, the

presence of hydrogen bond donor/acceptor sites or overlapping of p-orbitals in π-conjugated systems.

The differences in crystalline structures between polymorphs result in differences in their properties, of which solubility and dissolution rate are considered to be the most important. The solubility in aqueous media is of particular interest because it may influence bioavailability. Although a typical ratio of solubility of two polymorphs is less than two, it is not a rule. For example the ratio of two polymorphs of acetazolamide (I and II) is 1.11 [124], while for premafloxacin (I and III) or chloramphenicol (A and B) it exceeds 10 [125]. When the differences between the solubility of polymorphs are great, the minimum therapeutic concentration in the blood of the poorer soluble polymorph (according to the Hammond rule [126], the poorer soluble polymorph can be more therapeutically active) can be insufficient for its effective activity. It is a rule that anhydrates/ansolvates of API are better soluble than its hydrated/solvated forms.

There are a number of methods that can be used to characterise polymorphs of a drug substance [127] but the single crystal XRD is currently regarded as bringing definitive qualitative and quantitative evidence of polymorphism. Facility of XRD data acquisition and XRD usefulness as a screening technique make it is more often used than the other physico-chemical methods commonly used for the investigation of polymorphism, like hot-stage microscopy (melting point), solid-state infrared spectroscopy (IR), X-ray powder diffraction (PXRD), differential scanning calorimetry (DSC), thermogravimetric analysis (TGA), differential thermal analysis (DTA), FT-Raman spectroscopy and scanning electron microscopy (SEM). However, XRD analysis is not always possible, for example, when no single crystal of sufficient size, quality or stability can be obtained. The complementary tools of ever-growing value for the solid-state characterisation of API, excipients or drug formulations are the solid-state nuclear magnetic resonance spectroscopy (SS NMR) and a more sophisticated but less popular - nuclear quadrupole resonance (NQR). As compared to the IR, Raman, and XRD techniques, SS NMR or NQR are bulk techniques in which the particle size of the substance studied does not matter. In contrast to XRD, they can be applied even to amorphous samples (in the case of NQR after freezing in liquid nitrogen), although it is not easy because of great line widths. Both techniques are non-invasive and non-selective which means that low molecular weight components in the sample can be detected in a single experiment. In a sense they can be defined as selective because they allow detection of signals from the selected types of nuclei. Both techniques are quantitative, i.e. can be used to measure the content of API in a sample. Compared to other techniques, SS NMR and even more NQR are less sensitive and require more expensive equipment, which is their main drawback. Although in many cases SS NMR or NQR can be very effective, the majority of studies of pharmaceutical polymorphs are still performed in conjunction with other analytical techniques. While NMR has become in recent years very popular in studies of drugs, the use of NQR spectroscopy is rather scarce as from over 250 compounds whose polymorphism has been studied by this method [128] fewer than 20 were API, pharmaceutical formulations or excipients.

In general, the suitability of solid-state NMR or NQR for investigation of polymorphism follows from the fact that each polymorph is crystallographically different, which means that a certain nucleus in each polymorph is in a slightly different local molecular environment. Since the local environment at the nucleus site of interest is different, the SS NMR or NQR spectra differ slightly. In consequence, the isotropic chemical-shift / CSA tensor or NQR frequency/quadrupole coupling constant and asymmetry parameter derived

from these spectra also differ for the corresponding sites. Thus the analysis and spectral assignment of the solid-state NMR or NQR spectra of the polymorphs provides easy identification of polymorphic forms based only on the spectra recorded. More sophisticated analysis of the experimental solid-state NMR or NQR data often combined with modelling on the basis of quantum chemistry calculations (DFT or ab initio) can lead to the origin of differences in the structure of particular polymorphs.

For the first time the differences in the SS NMR spectra being a result of polymorphism have been reported by Byrn et al. for benoxaprofen (non-steroidal anti-inflammatory drug), nabilone (antiemetic, an adjunct analgesic for neuropathic pain) and pseudo-polymorphism of cefazolin (first-generation cephalosporin antibiotic broad-spectrum) [129]. The method of choice was then PXRD, but surprisingly the solid-state ^{13}C CP/MAS NMR spectrum of each polymorph was found to be distinctly different. These studies performed on bulk API material initiated more systematic studies of polymorphism by SS NMR. The first NQR studies of API polymorphism have been reported much earlier than those by SS NMR [130,131]. The first studied API was chloral hydrate (trichloroacetaldehyde monohydrate, 2,2,2-trichloro-1,1-ethanediol), one of the oldest synthetic agents, a sedative and hypnotic drug. Significant improvement in sensitivity and resolution meant that both methods have become important complementary tools in the studies of polymorphism. SS NMR has been recently accepted by US and Japan Pharmacopeia as a method for polymorphism determination, NQR studies having been too scarce and not so popular.

3.2.1.1 Packing polymorphism

The packing polymorphism occurs when rigid molecules of the same conformation are packed in different ways in a crystal.

Glycine

An interesting example and the most intensively studied organic polymorph, is glycine, the simplest amino acid, a precursor to proteins, an inhibitory neurotransmitter in the central nervous system, anti-cancer, chronic multiple sclerosis and schizophrenia drug, also widely applied as excipient to drugs. In the solid state, glycine crystallizes in three forms α, β and γ, which can be distinguished by XRD [132-134] or neutron diffraction [135,136]. The α form is stable, the β form is unstable and readily transforms to α [132], while the γ form is stable at room temperature, but irreversibly converts to α upon heating above 438K. The internal arrangement of molecules in layers is the same for α, β, which crystallize in monoclinic, P21/n and P2$_1$, respectively, while γ crystallizes with a trigonal hemihedral symmetry, in hexagonal, P3$_1$. In general, in all three forms the glycine molecules are zwitterions, the hydrogen-bonded double layers of molecules are packed by van der Waals forces and the only difference in the two forms results from the nature of the hydrogen bonding. Polycrystalline samples can be distinguished using X-ray powder diffraction [138] or infrared (IR) spectroscopy [133, 139]. The well-marked differences in the N–H stretching frequency in IR spectra (3164 cm^{-1} for α-glycine, 3191 cm^{-1} for β-glycine, and at 3105 cm^{-1} for γ-glycine) allow fast identification of the polymorphs in a polycrystalline sample [140].

The polymorphs of glycine can be also distinguished using solid ^{13}C NMR or ^{15}N NMR by a direct comparison of the isotropic chemical shifts and/or chemical shift tensor components [139, 141]. However, the differences in these values are relatively small, at most of an order of 1ppm, Table 1. The sources of differences in the components of the chemical shift tensor, especially σ_{yy} at ^{13}C and σ_{zz} at ^{15}N for polymorphs have been revealed by the quantum

chemistry methods, DFT [142]. These differences have been explained by changes in the hydrogen bonding arrangement of α-glycine and β-glycine. In addition, polymorphs can be readily identified by the spin-lattice relaxation times [1]H $T_{1\rho}$ and [15]N T_1 , which are much shorter for α-glycine and β-glycine than for γ form.

SITE	FORM I α					FORM II β					FORM III γ				
	σ_{iso} [ppm]	σ_{xx} [ppm]	σ_{yy} [ppm]	σ_{zz} [ppm]	T_1, $T_{1\rho}$ [s]	σ_{iso} [ppm]	σ_{xx} [ppm]	σ_{yy} [ppm]	σ_{zz} [ppm]	T_1, $T_{1\rho}$ [s]	σ_{iso} [ppm]	σ_{xx} [ppm]	σ_{yy} [ppm]	σ_{zz} [ppm]	T_1, $T_{1\rho}$ [s]
[13]C	176.5 177.0 176.5	243.5 244	178.9 180	108.7 105	12 , 0.18 (C=O) 11, 0.043 (NCH$_2$)	175.5 175.4	243.5	176.5	106.0	8, 0.012 (C=O) 2.3, 0.30 (NCH$_2$)	174.5 174.9 174.6	243.2 243	174.9 173	106.6 108	55, 0.27 (C=O) 22, 0.053 (NCH$_2$)
[15]N	32.3 32.5	40.2	33.1	24.3	0.240 0.93	-	-	-	-	-	32.9 32.8	40.5	33.6	26.7	0.150 0.018

Table 1. The isotropic chemical shifts and CSA tensor components, spin-lattice relaxation times T_1, $T_{1\rho}$ - in glycine forms α, β and γ [140, 141].

The use of ^{14}N NQR for differentiation of polymorphs is even much easier than the use of NMR, as the frequencies (v), quadrupole coupling constants e^2Qq/h and asymmetry parameters (η) for β and γ-glycine, are significantly higher than for α - form, Table 2. The changes in quadrupole coupling constants and asymmetry parameters reflect the differences in the hydrogen bonding length pattern.

FORM I α					FORM II β					FORM III γ				
v_+ [MHz]	v_- [MHz]	v_0 [MHz]	e^2Qq/h [MHz]	η [-]	v_+ [MHz]	v_- [MHz]	v_0 [MHz]	e^2Qq/h [MHz]	η [-]	v_+ [MHz]	v_- [MHz]	v_0 [MHz]	e^2Qq/h [MHz]	η [-]
1.100	0.785	0.315	1.257	0.501	1.133	0.843	0.290	1.317	0.440	1.044	0.822	0.222	1.244	0.357
0.988	0.705	0.283	1.129	0.501			-	-	-			-	-	-

Table 2. [14]N NQR frequencies, quadrupole coupling constants and asymmetry parameters - in glycine forms α, β and γ [143,144].

Acetazolamide

An example of packing polymorphism is acetazolamide, the first non-mercurial diuretic drug, used clinically as antiglaucoma, antiepileptic or antiulcer, benign intracranial hypertension, altitude sickness, cystinuria and dural ectasia drug. Acetazolamide crystallizes in two polymorphic forms I and II [145], which differ in the spatial molecular arrangement and hydrogen bonding pattern, but not in the conformation of molecules. Thus the polymorphism of acetazolamide is classified as a packing type or pure combining association type. Interesting results concerning the interaction pattern have been obtained using NMR, NQR and DFT/QTAIM calculations [104]. The marked differences in the ^{14}N NQR frequencies, quadrupole coupling constants and asymmetry parameters at the nitrogen sites in the thiadiazole ring and -NH$_2$ on going from phase I to phase II allow distinction of both polymorphs, Table 3. Surprisingly the changes in the NQR parameters are negligible at -NH site. Moreover, the change in the parameters at the –NH$_2$ nitrogen site is significant.

As the source of this diversity can be either different hybridisation or participation in different hydrogen bonds or other weak intermolecular interactions, a detailed examination

of the closest-neighbourhood of each site in acetazolamide molecule was necessary. The XRD data were available but they could not explain the differences. Therefore each form was analysed within DFT/QTAIM, which shed some light on the bonding pattern in both polymorphs, Fig. 2. Table 3 [104].

Form	Nitrogen position	NQR					T [K]	DFT B3LYP/6-311++G(d,p)*	
		v_+ [MHz]	v_- [MHz]	v_0 [MHz]	e^2qQ/h [MHz]	η [-]		e^2Qq/h [MHz]	η [-]
I	-NH$_2$	3.320	2.280	1.040	3.730	0.557	295	4.378	0.578
	-NH-	2.830	1.930	0.900	3.170	0.567		3.400	0.833
	N(4)	3.650	3.040	0.610	4.460	0.274		5.231	0.154
	N(3)	3.520	2.400	1.120	3.950	0.568		4.247	0.719
II	-NH$_2$	3.555	2.515	1.040	4.045	0.514	210	4.321	0.678
	-NH-	2.840	1.940	0.900	3.190	0.565		3.375	0.827
	N(4)	3.660	3.070	0.590	4.490	0.263		5.218	0.166
	N(3)	3.570	2.510	1.060	4.050	0.523		4.263	0.706

* cluster of 14 (form I) and 6 (form II) molecules

Table 3. ^{14}N NQR frequencies, quadrupole coupling constants and asymmetry parameters - in acetazolamide form I at T = 295 K and acetazolamide form II at 210 K.
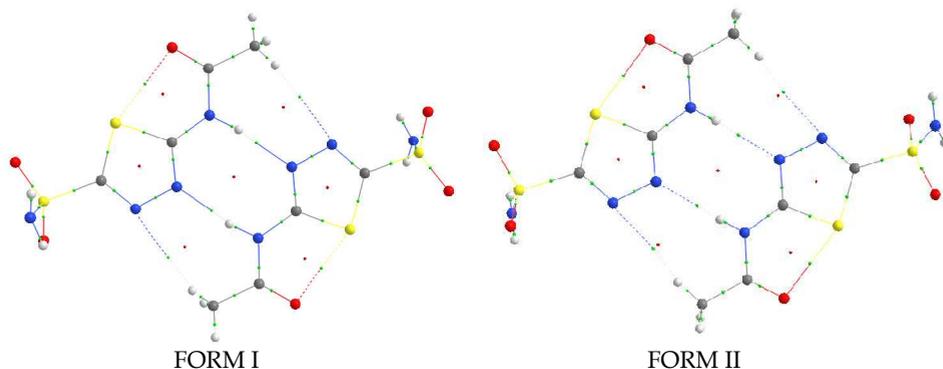

FORM I   FORM II

Fig. 2. Polymorphic structures of acetazolamide.

In form I an –NH$_2$ group (proton donor) forms a relatively strong NH…N hydrogen bond of 3.080 Å in length with the nitrogen N(4) from the thiadiazole ring (hydrogen bond acceptor), i.e. that which does not participate in the formation of the molecular pair mentioned earlier. The additional two hydrogen bonds, NH…O, of 2.958 Å and 3.026 Å in lengths, are formed by the oxygen from a sulfone group as a hydrogen bond acceptor and N from -NH$_2$ group as a hydrogen bond donor. In form II, the second of the two atoms in the 1,3,4-thiadiazole ring, N(4) (hydrogen bond acceptor) forms a very weak CH…N hydrogen bond of the length 3.695 Å at 93.2K with the carbon atom from -CH$_3$ (hydrogen bond donor). Besides, two sulfonamide groups are bound by two identical N-H…O hydrogen bonds of the lengths 2.988 Å and energy -20.8 kJ/mol at 93.2 K. Each -NH$_2$ group forms also a bit stronger hydrogen bond NHO with a neighbouring acetyl group, of the length 2.896 Å and with the energy -28.4 kJ/mol at 93.2 K, Table 4..

FORM	BOND	ρ [a.u.]	Δ(ρ) [a.u.]	ε [-]	E [kJ/mol]
I RT Proton opt.	N(4)…HC	-	-	-	-
	N(3)H….N(acetyl)	0.0319	0.084745	0.0727	-29.49
	S1…O(acetyl)	0.0258	0.082293	0.0035	-24.20
II 93.2 K Proton opt.	N(4)…HC	0.0069	0.0211	0.0934	-4.72
	N(3)H….N(acetyl)	0.0408	0.1014	0.0682	-44.06
	S1…O(acetyl)	0.0200	0.0630	0.0510	-18.51
I RT X-Ray	N(4)…HC	0.0034	0.0115	2.3682	-2.55
	N(3)H….N(acetyl)	0.0182	0.0723	0.1108	-15.01
	S1…O(acetyl)	0.0259	0.0823	0.0038	-24.22
II 93.2K X-Ray	N(4)…HC	0.0042	0.0134	0.1359	-2.96
	N(3)H….N(acetyl)	0.0325	0.1021	0.0827	-33.12
	S1…O(acetyl)	0.0202	0.0622	0.0612	-18.22

Table 4. Topological parameters of ρ(r) for acetazolamide (the electron density at BCP (ρ_{BCP} (r), its Laplacian $\Delta BCP(\rho)$, the ellipticity of the bond (ε) and estimated hydrogen bonding energy according to Espinosa (E_E) calculated at the B3LYP/6-311++G(d,p) level of theory).

Thus the largest differences in the symmetry of electron distribution between I and II forms should be observed at the -NH$_2$ site. Indeed, the asymmetry parameter for -NH$_2$ site is higher in I than in form II because in form I the nitrogen atom from -NH$_2$ participates in three different intermolecular interactions, while in form II it participates only in two interactions. For the same reason e^{2}Qqh^{-1} is smaller at -NH$_2$ site in polymorph I in comparison to that in form II. Differences in bonding schemes of the terminal -NH$_2$ group in the two crystallographic modifications are found to be responsible for a significantly different ^{14}N NQR parameters, which can be used as a measure of the strength of hydrogen bonds [104].

Cloxiquine

An unusual case of packing polymorphism has been noted for cloxiquine (5-Chloro-8-hydroxyquinoline) API of antibacterial, antifungal, antiaging and antituberculosis drugs. In the solid state, cloxiquine crystallizes in two forms I and II differing in the arrangement of rigid molecules. Both forms can be easily distinguished by XRD [145]. In both forms the hydroxyl hydrogens are capable of forming a multicentre i.e. bifurcated O-H...N hydrogen bonds, one intramolecular and the other intermolecular, which simultaneously lead to formation of five-membered hydrogen-bonded chelate rings [N, C(9), C(8), O, H(8)] and to dimerisation of the molecules. Such patterns, usually termed supramolecular synthons [146], are according to XRD data, independent of the polymorphic form, however in the dimeric structure of cloxiquine form I the paired molecules in the units are twisted, while in form II they are not. Due to this subtle difference in the planarity of dimeric structures which consist of the paired molecules linked by bifurcated hydrogen bonds in the units - twisted in form I, and planar in form II [146], accompanied by change in the hydrogen bond lengths, the structure units can be differently packed to yield the two polymorphs. XRD indicated that the supramolecular synthons expand into a column by π-π stacking interactions, along the crystallographic c axis in I and b axis in II [146]. The columns constructed in this way are stabilized in the 3D crystal structure by Van der Waals forces.

The pronounced differences in ^{14}N and ^{14}O NQR spectra (frequency shifts: 0.175, 0.156, 0.020 and 0.107, 0.234, 0127 MHz, respectively) but only slight in ^{35}Cl (frequency shifts by 0.033MHz) allows distinction between forms I and II [148]. The elongation of the hydrogen bond in form I in comparison to form II is reflected by a change in the asymmetry

parameter. DFT/QTAIM formalism was applied to study supramolecular synthon pattern in detail and estimate the strength and character of hydrogen bonds. The intramolecular N-H…O bonds in cloxiquine form II are weak and slightly stronger than the intermolecular N-H…O bonds, but generally weaker than typical and mainly electrostatic ones, while intermolecular N-H…O bonds in cloxiquine form I are moderate and partially covalent in nature. The large differences in strength of the corresponding H-bonds in both cloxiquine forms, suggest the interplay in H-bonds linking adjacent molecules in dimers, Fig. 3. The strong interactions between monomers being components of supramolecular synthon of cloxiquine form II, i.e. the two competitive (intra- and intermolecular) O-H...N hydrogen bonds, the former slightly stronger than the latter, and an additional intermolecular hydrogen bond C-H..O, which in contrast to form I links atoms in the same dimer and thus is more than twice stronger, are responsible for drastic differences in polarity of supramolecular synthons and result in the planarity of form II. Moreover, the strength of stacking $\pi \cdots \pi$ interactions is found to be strongly dependent on planarity thus they are much weaker in form I than in form II of cloxiquine. The NQR parameters have been correlated with both the molecular density of the packing and the distance between stacking layers, as a consequence of different hydrogen bonding strength and different $\pi \cdots \pi$ overlaps [148]. Both forms differ in water solubility and dissolution rate (form II is 1.47 times better soluble than form I) [146].
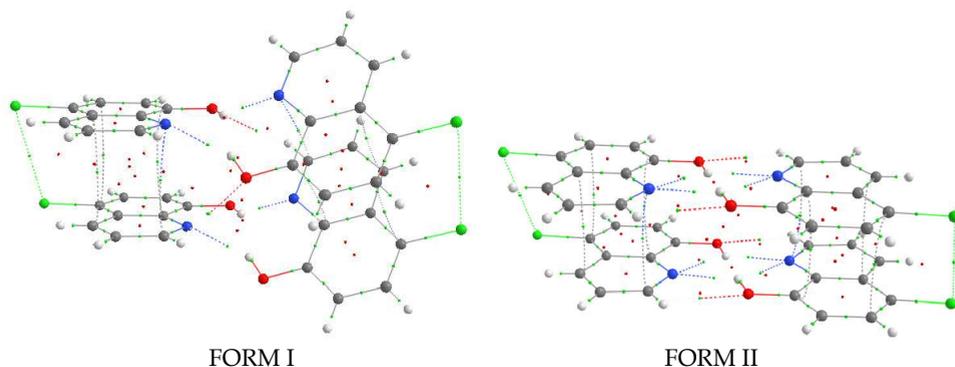

FORM I   FORM II

Fig. 3. Polymorphic structures of cloxiquine.

3.2.1.2 Conformational polymorphism

Conformational polymorphism occurs when flexible molecules with different conformations are packed in different ways in the same crystal unit.

The group of sulfonamides, of well-known antibacterial activity, make a good illustration of conformational polymorphism. These compounds have been more or less systematically studied since their discovery in the late 1930s. Till 1948 more than 5,000 derivatives have been synthesised, among which several drug classes have been identified (e.g. antibacetrial, hypoglycemics, carbonic anhydrase inhibitors, saluretics, and tubular transport inhibitors). The antibacterial effectiveness of sulfonamides, classified as the sulfa drugs, is determined by a highly polar sulfonyl group, which is responsible for the similarity of sulfonamides to the p-aminobenzoate ion. Their intermolecular interactions patterns have some common features i.e. amido protons show a greater preference for hydrogen bonding to amidine

nitrogens and cocrystal guests, whereas the amino protons show a greater preference for hydrogen bonding to sulfonyl oxygens [149]. The dominant hydrogen-bond pattern is a chain with an eight atom repeat unit. Molecules of sulfonamides are extraordinarily versatile due to conformational (amine and amide) and hydrogen-bonding capabilities. Thus sulfonamides reveal the ability to crystallize in multiple solid-state structures i.e. exhibit polymorphism. In fact many polymorphs and hundreds of solvates have been discovered for this class of compounds [102].

Sulfanilamide

The most intensively studied compound from this class is sulphanilamide (4-aminobenzenesulfonamide) - a sulfonamide antibiotic used in treatment of streptococcal infections. In the solid state, it crystallizes in four forms α, β, γ, [149-151] and δ [152-154] fully investigated by XRD. The different d-spacing of three strongest XRD diffraction lines (4.91, 6.57, 3.57; 6.12, 4.49, 4.23; and 3.90, 3.78, 33.6 for α, β, γ, respectively) allows easy distinction of each polymorph. The form stable at room temperature is β- sulfanilamide. The remaining forms gradually revert to this form on storage. On slow heating, β-sulfanilamide slowly transforms to α and then to γ. The forms β and γ are considered to be enantiotropically related. The origins of polymorphism of sulfanilamide were found to be connected with the versatile hydrogen-bonding capabilities [154]. The differences in the chemical shifts at ^{13}C and at ^{15}N permitting the distinction between individual polymorphs Table 5, [156] are of the same order as in glycine. However, the chemical shifts at N(1) site were almost the same for the three polymorphs. The spin-lattice relaxation times [1]H T_1 and [1]H $T_{1\rho}$ allow distinction of **β**-sulfanilamide from the other polymorphs, while [15]N $T_{1\rho}$ allows distinction of all three polymorphs and what is more, point to substantial differences in mobility around N(1) and N(2) sites. This result has pointed to different molecular mobility in the solid-state at each site in each polymorph.

ISOT.	SITE	FORM I α				FORM II β				FORM III γ			
		σ_{iso} [ppm]	ζ [ppm]	η [-]	T_1, $T_{1\rho}$	σ_{iso} [ppm]	ζ [ppm]	η [-]	T_1, $T_{1\rho}$	σ_{iso} [ppm]	ζ [ppm]	η [-]	T_1, $T_{1\rho}$
[13]C	C(1) C(4) C(2),C(6) C(3),C(5)	153.7 128 113.1, 115.3 128.3	-	-	-	153.4 127.1 112.3,117.1 129.5	-	-	-	151.0 127.1 112.7, 115.1 129.6	-	-	-
	C(1) C(4) C(2),C(6) C(3),C(5)	166.5,157.6 134.5 118.8, 124.0 134.5	-	-	-	165.5, 156.0 135.6 118.3,123.1 133.3	-	-	-	165.5, 156.0 137.1 120.0, 122.5 135.0	-	-	-
[15]N	N(1) N(2)	-312.2 -288.8	±46 70	1 0.4	0.64ms 6.5ms	-312.1 -284.1	43 71.2	0.8 0.4	0.64 ms 10ms	-312.1 -280.6	±45 67.3	1 0.3	1.5 ms 4.2ms
[1]H	--	-	-	-	94 s 7.2 s	-	-	-	8 s 7.1 s	-	-	-	82 s 3.0 s

Table 5. The isotropic chemical shifts and CSA tensor components - in sulfanilamide forms α, β and γ [156].

The pronounced differences in ^{14}N NQR spectra, Table 6, [157] make the differentiation of α and β polymorphs of sulfanilamide much easier than with the use of SS NMR. The shifts in

frequencies (v) and differences in quadrupole coupling constant e^2Qq/h and asymmetry parameter (η) with reference to those of α form (0.555, 0.522, 0.410 and 0.693 MHz) are evident.

FORM I α			FORM II β			T [K]
v [MHz]	e²Qq/h [MHz]	η [-]	v [MHz]	e²Qq/h [MHz]	η [-]	
3.460 2.527 0.933	3.990	0.47	2.905 2.005 0.900	3.270	0.55	210
3.115 2.603 0.512	3.810	0.27	2.705 1.910 0.790	3.080	0.51	255

Table 6. ^{14}N NQR frequencies, quadrupole coupling constants and asymmetry parameters - in sulfanilamide form I at T = 295 K and form II at 210 K [156].

Thus NQR seems an even more suitable technique for identification of polymorphs and studies of changes in the intermolecular pattern due to polymorphic transitions.

While the number of drugs studied by solid state NMR is large, ^{14}N NQR was successfully applied for the studies of a group of sulfonamides exhibiting confomational polymorphism including sulfapyridine (4-amino-*N*-pyridinylbenzene sulfonamide), sulfadiazine (4-amino-*N*-pyrimidin-2-yl-benzenesulfonamide), sulfamerazine (4-amino-*N*-(4-methylpyrimidin-2-yl)benzenesulfonamide), sulfamethazine (4-amino-*N*-(4,6-dimethylpyrimidin-2-yl)benzene-sulfonamide), sulfathiazole (4-amino-*N*-(1,3-thiazol-2-yl)benzenesulfonamide)[157]. Interesting results have been also obtained for non-steroidal anti-inflammatory drug piroxicam ((8*E*)-8-[hydroxy-(pyridin-2-ylamino)methylidene]-9-methyl-10,10-dioxo-10λ⁶-thia-9-azabicyclo[4.4.0]deca-1,3,5-trien-7-one) [158]. ^{35}Cl NQR was successfully applied in the study of chloral hydrate (trichloroacetaldehyde monohydrate, 2,2,2-trichloro-1,1-ethanediol), one of the oldest synthetic agents of sedative and hypnotic effects [130], non-steroidal anti-inflammatory diclofenac (2-[2-(2,6-dichlorophenyl) aminophenyl] acetic acid) [159], anti-diabetic chloropropamide (1-(4-chlorophenyl)sulfonyl-3-propyl-urea) [159], diuretic furosemide (4-chloro-2-furfurylamino-5-sulfamoyl-benzoic acid) [160]. Differentiation of the polymorphic forms of these compounds is not difficult because of a distinct shift in the ^{14}N-NQR or ^{35}Cl NQR frequency (0.65, 0.425, 0.58 and 0.18MHz for sulfamerazine, 0.23, 0.48, 0.42, 0.30, 0.10 and 0.08MHz for sulfathiazole, and 0.142MHz for furosemide; 0.287MHz for chloral hydrate, 0.176MHz for diclofenac, 0.306 MHz for chloropropamide) while, at the accuracy of determination of the order of 0.001 MHz (^{14}N NQR) and 0.010 MHz (^{35}Cl NQR) even for broad lines.

Apart from typical NQR parameters, polymorphs are often readily identified (similarly as in NMR study) on the basis of changes in the NQR linewidth (1.5kHz for sulfapyridine, 10kHz for chloropropamide) or in the spin-lattice (sulfapyridine 70-fold, chloropropamide 2-fold) or spin-spin (chloropropamide 10%) relaxation times. However, sometimes the estimation of the amount of polymorphs in a mixture on the basis of these parameters can be misleading [156].

It is known that in some cases SS NMR was helpful in explaining contradictions concerning polymorphism following from IR, XRD and DSC studies [118]. A briliant example indicating

the usefulness of SS NMR is cefinidir, a broad-spectrum antibiotic, proven effective for common bacterial infections of the ear, sinus, throat, and skin. The use of XRPD and IR was not sufficient and Abbott's 'new polymorph' discovered using these techniques in 2003 was proved to be not a polymorph, but a pyridinium salt. [118]. Up to now no examples of such spectacular NQR applications had been given, but it does not mean that the potential of this method will not be exploited for this purpose in future.

Solid-state NMR or NQR can be powerful methods for comparing physical forms of the drug substance after pharmaceutical processing or manufacturing. Variation in the chemical shift or NQR frequency from polymorph to polymorph allows identification of differences in the atomic environment. In addition, NMR and NQR are quantitative analytical techniques, i.e. the intensity of their respective signals is directly proportional to the number of nuclei in the sample [158,160,161]. Thus it is possible to estimate the quantitative polymorphous composition or a relative proportion of polymorphs in a sample and the result is reliable. NMR is an important spectroscopic tool for the study of solid-state drug formulations. NQR method has such a potential. Both methods can be used for analysis of pure API or mixtures of polymorphs in pure drug substance and in dosage formulations. A detailed analysis of this aspect of NMR or NQR applications is beyond the scope of this chapter.

3.2.2 Tautomerism

Tautomerism (Greek: *tautos-identical*; *isomerism - Laar, 1985* [162]) can be defined as a reversible structural isomerism involving the sequential processes of bond cleavage, skeletal bond migration and bond reformation. Tautomerisation plays an important role in mutations or enzymatic reactions. Thus the inspection of the most stable tautomeric forms allows further discussion of the functional implications of tautomerism for recognition and binding to DNA, proteins or enzymes**.** Many purines and pyrimidines occur in tautomeric forms whose relative stabilities depend on the environment. Any alteration or modification in the base pairing scheme of DNA due to the existence of different tautomeric forms also may result in perturbation to the replication process or spontaneous mutations, i.e. to a reduced stability of DNA. The presence of an abnormal tautomer can be even more deleterious to the stability of DNA. On the other hand, these unique capabilities of modifying DNA may be relevant for the process of designing of DNA binding drugs of distinct antitumor efficacy. The fact that often the less abundant tautomer is the most reactive one (Hammond rule [126]) further reinforces the relevance of investigation of intermolecular interactions in the solid state. The preference for different tautomeric forms is a clear indication of the importance of intermolecular interactions, in particular H-bonding, in determination of the structure of the condensed phase.

Polyhalobenzimidazoles

An example are the studies of annular tautomerism of polyhalobenzimidazoles, Fig.4, [105]. NQDR and DFT results have suggested the presence of prototropic annular tautomerism 50:50, which is in a good agreement with the XRD and ^{1}H-NMR data. According to the XRD data, all halogenobenzimidazole crystals are isostructural and have the same space group $I4_1/a$. The intermolecular hydrogen bonds N1-H···N3' and N1'···H-N3 which link each molecule with two neighbouring ones, occur between the molecules related by the fourfold screw symmetry axis along the c direction of the tetragonal cell, Fig. 4. According to QTAIM analysis, the N-H···N bonds formed by both nitrogen atoms (N1 and N3) differ

insignificantly in strength (by less than 0.4 kJ/mol), which is in a good agreement with the differences in their lengths.

Comparison of the HB strength and electron density at the hydrogen bond critical points in a group of polyhalogenobenzimidazoles suggests that hydrogen bonds are of similar strength. The differences in e^2Qqh^{-1} and η observed for both nitrogen sites suggests that hydrogen bonds are asymmetric and should be described by a proton double minimum potential, which is confirmed by the QTAIM analysis. As the compounds crystallise in the centrosymmetric group, the polar chains of the molecules arranged in one direction are accompanied by antiparallel chains arranged in the opposite direction and this spatial ordering is very close to that observed in the **α** form of 1H-benzimidazole. This suggests that a packing polymorphism similar to that in 1H-benzimidazole can be expected in polyhalogenobenzimidazoles. Additionally QTAIM calculations have revealed weak intermolecular interactions in polyhalogenobenzimidazole structures i.e. N···X (X = H, Cl, Br or I) and two kinds of Y···Z (Y, Z=Cl, Br or I) contacts, which are much weaker than hydrogen bonds. The local potential energy density at BCPs indicated the following ordering of the intermolecular interactions according to increasing bond strength (E) : N1-H···N3 ~ N1-H···N3 < N1···X < X···Y (X, Y from the same ring) < X···Y (X, Y from the different rings). The pattern of intermolecular contacts has been found responsible for the specific crystal arrangement and the annular prototropism [105].
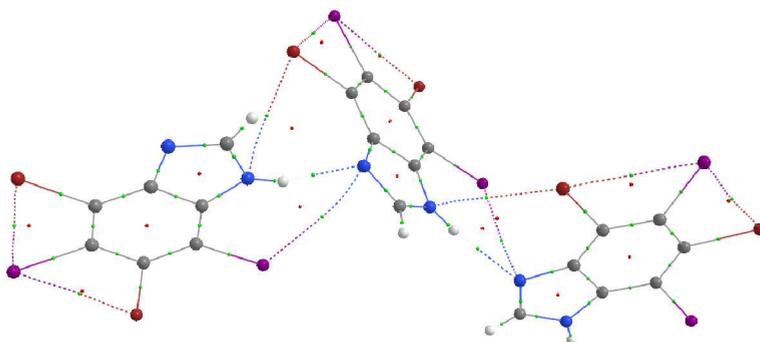

Fig. 4. The intermolecular hydrogen bonds pattern in polyhalogenobenzimidazoles.

2-thiocytosine and cytosine

The solid-state tautomerism studies are well illustrated by those performed for canonical natural nucleic acid base cytosine, and its analogue 2-thiocytosine. The replacement of cytosine by 2-thiocytosine may perturb base-pairing process of DNA or it can produce spontaneous mutations i.e. reduced stability of DNA. The compound of 2-thiocytosine is a potential anti-leukaemic and anticancer agent [163,164]. Numerous derivatives including 1-(β-D-arabinofuranosyl)-2-thiocytosine and its analogues and complexes with trimethyl platinum have been synthesized and their enzymatic reactivity and antitumor activity have been studied [165]. The cytotoxic activity of some of them has been found even higher than that of cisplatin and was manifested even against cisplatin resistant cell lines [165]. The arrangement of molecules in the crystals of 2-TC is essentially the same as that found for cytosine, but more complicated due to the existence of two inequivalent molecules in the elementary cell, Fig. 5, [166].

In the 2-thiocytosine crystalline structure, supramolecular synthons (dimers) are linked to the neighbouring supramolecular synthons by intermolecular interactions of the same pattern i.e. weaker H-bonds: N(1)-H(1)...N(3') and N(4')-H(4')...S(2) of 3.114 and 3.408 Å. Neighbouring ribbons are linked together by much longer, thus considered weak, N-H...S bonds of the lengths 3.466 and 3.551 Å [166]. The application of QTAIM allows detection and distinction of many weaker interactions in the crystalline structure of 2-thiocytosine [167], which was not possible in a standard X-Ray study [166]. Indeed in the crystalline structure of 2-thiocytosine, the QTAIM analysis has revealed the presence of four varieties of intermolecular interactions not involving hydrogen atoms (N…N, N…C and N…S), apart from the hydrogen-bonds (N-H…N, N-H…S, C-H…S and C-H…C).

2-THIOCYTOSINE   CYTOSINE
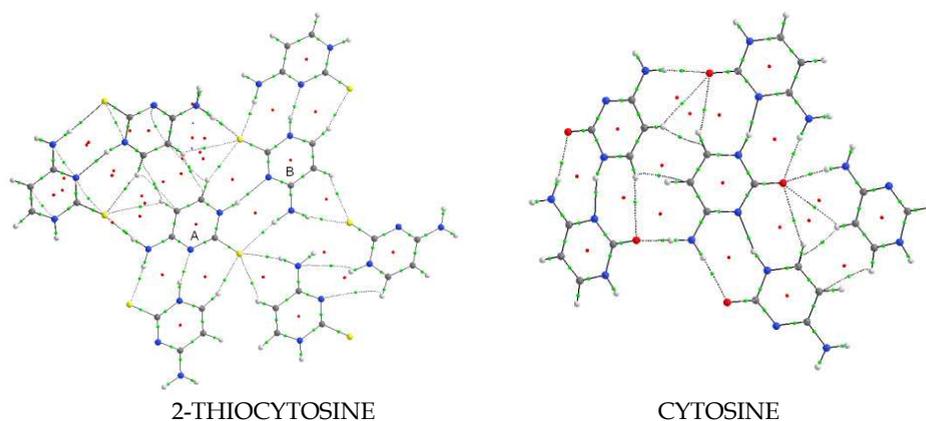
Fig. 5. Arrangement of molecules in the crystals of 2-thiocytosine and cytosine.

In general, the hierarchy of structures in crystals of 2-thiocytosine molecules-dimers-ribbons-stacks is reflected by the progressively weaker bonds. The N(1')-H(1')…N(3) interaction is found to be the strongest (-23.18 kJ/mol), followed by N(4)-H(4)...S(2') (-17.13 kJ/mol). The third H-bond linking adjacent molecules into a dimer C(6)-H(6)…S(2'') of 3.532 Å (-9.43 kJ/mol) is much weaker and thus not revealed by X-ray [165]. The hydrogen bonds linking adjacent supramolecular synthons are: N(1)-H(1)...N(3') of 3.114 Å and -16.10 kJ/mol, N(4')-H(4')...S(2) of 3.408 Å and -13.95kJ/mol. The H-bonds linking neighbouring ribbons are: N(4)-H(4)...S(2') of 3.466 Å (-3.17 kJ/mol) and 3.551 Å (-8.01 kJ/mol) and C(5)-H(5)...S(2') bond of length 4.037 Å (-2.85 kJ/mol), two C(5)-H(5)...C(6') and C(5')-H(5')...C(6) of 3.688 Å (–3.99 kJ/mol) and 3.943 Å (-1.91 kJ/mol), respectively. Additionally between layered dimers a few π-π interactions involving non-H atoms, purely van der Waals in nature, were detected: N(1)…N(3''), N(1)…C(5'') and N(3)…S(2'') of the lengths 4.094, 3.411 and 3.516 Å and roughly estimated energies -1.14, -4.06 and -4.01 kJ/mol [167]. It is worth noting that N(4) from the NH_2 group, in contrast to N(1) and N(3), does not participate in this stacking π-π interactions. The differences between the isosurface representations of Laplacian at nitrogen sites in both inequivalent molecules are small, however they suggest that –NH- site exhibits more symmetrical electron density distribution in molecule A than B, while –N= and -NH_2 sites exhibit more symmetrical distribution in molecule B than A, which is in a good agreement with experimentally obtained results [167].

3.2.3 Stability

The term 'stability' of drugs refers to their chemical and physical integrity and can be influenced by many factors (temperature, light, air, humidity and pressure), as well as the package components. In the pharmaceutical context the influence of temperature and /or irradiation is of particular interest. These factors can lead to the transition to undesirable meta-stable polymorph or degradation products, which means reduction of bioavailability, loss of activity or even toxicity.

3.2.3.1 Thermal stability

Solid state NMR and NQR are particularly well suited for fast monitoring of temperature effect on the thermal stability of API. Temperature studies of chemical shifts on different isotopes, or ^1H NMR second moment or ^{35}Cl or ^{14}N NQR frequencies or different spin-lattice relaxation times (r.g. $T_1(^1H$ NMR), $T_1(^{35}Cl$ NQR) or $T_1(^{14}N$ NQR)) provide the information on the compounds stability, including the phase transitions (changes in the crystallographic structure and ordering). For many vitamins and drugs, the temperature dependencies of second moment of ^1H NMR or spin-lattice relaxation time have been studied along with molecular motions - hindered rotations, reorientations, proton transfer, but the pharmaceutical context has been neglected. Similarly, for a few APIs including urotropine (HMT), an antiseptic used for the prophylaxis of urinary tract infections, or chloral hydrate - a local anaesthetic, the temperature dependencies of NQR frequency and phase transition have been studied more or less systematically [168-170], but not in the pharmaceutical context.

The thermal stability aspect most important and interesting from the pharmaceutical point of view is the phase transitions of APIs, especially the uncontrolled ones. The unstable polymorphs often convert into more stable ones by phase transitions. Uncontrolled polymorph transitions of API may happen during the final crystallisation, storage in the parent solution, drying, wet granulation, micronization, tablet pressing, or even in shelf life (storing). In general two types of polymorphous transitions are distinguished the enantiotropic and the monotropic ones. The enantiotropic transition occurs at a specific temperature at which the originally more stable polymorph transforms into another finally stable polymorph. Transitions of this type are often reversible and well-defined. The second type of polymorphous transitions, the monotropic one does not occur at a specific temperature in solid state. The polymorph transition passes over the liquid phase, which means crystallisation from a different solvent. Most of polymorphous transitions of APIs are monotropic and not enantiotropic which is a negative phenomenon.

Interesting results have been obtained for hydrochlorothiazide (HCTZ), a diuretic drug applied to beat high blood pressure and heart failure. Among the DTA, NMR, NQR and XRD methods [171,172] the most sensitive to its phase transitions was NQR [172]. The anomalies in the temperature dependence of the ^{35}Cl NQR frequency for HCTZ, the change in the linewidth (being a result of an increase in the spin-lattice relaxation time), small but notable changes in the slope and the jump in frequency observed at 253 K, not exceeding 0.05 MHz, together with the lack of hysteresis (which distinguishes the first- and second-order transitions) indicated a second-order transition. This transition was not revealed by the temperature dependencies of the ^1H second moment or ^1H spin-lattice relaxation time (NMR), which were able to detect only trans-gauche jumps of $-NH_2$. Moreover, the phase transition clearly visible in NQR was only weakly indicated by the thermal dependence of heat capacity (DTA measurements) [171]. This phase transition was interpreted as a change

in the structural ordering connected with the reorientation of the -NH$_2$ group in a specific temperature in solid state i.e. the enantiotropic one. Indeed, in a short time after detection of the conformation phase transition, the polymorphs of HCTZ, Fig. 6, have been crystallised and their structure by XRD have been resolved [173]. Thus the NQR studies contributed to the evidencing of polymorph transforms and understanding of the nature of phase transition.
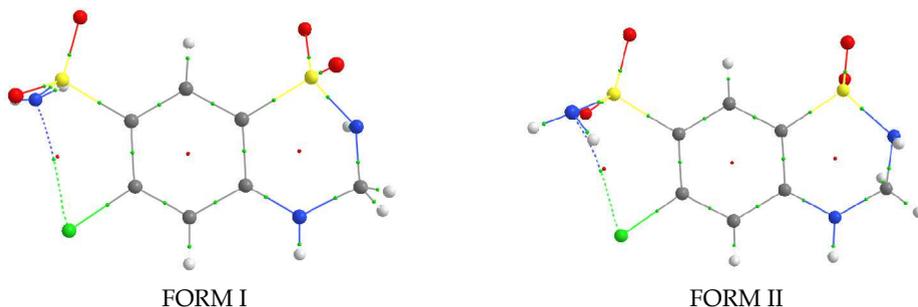

FORM I   FORM II

Fig. 6. Polymorphic structures of hydrochlorothiazide.

Another interesting case is chloropropamide for which NQR was able to detect not only the A to C phase transition, but also different molecular dynamics manifested as a difference in the slope of the temperature dependencies of NQR frequencies for A and C polymorphs [159]. Temperature dependencies of NQR frequencies can also bring information on the stability of a compound studied. For instance, a smooth temperature dependence of NQR frequency for triclosan suggests that its ring maintains almost the same conformation in the temperature range studied (no ring rotation), which has been confirmed by XRD results [98]. One more interesting example illustrating the potential of molecular dynamic NQR studies are the results obtained for antipsychotic and antihistaminic drug – phenothiazine [174]. [14]N NQR frequencies measured in both the low- and high-temperature phases indicated the presence of large thermal librations of the phenothiazine molecules in both crystallographic phases as well as an order-disorder phase transition associated with reorientations of the quite rigid phenothiazine molecules [175] around the orthorhombic axis. The quadrupole coupling constant have not been affected by the phase transition, while the asymmetry parameter reflected this phase transition even more evidently than the ^{14}N NQR frequencies. Thus the NQR studies contributed to the understanding of the microscopic mechanism of the phase transition.

3.2.3.2 Photostability

In the solid state, the temperatures required for different types of degradation differ, thus the mechanisms of thermal and photochemical degradation are expected to be different. Because light irradiation of drugs is known to have a very strong, often negative effect on their activity, the integral part of research on API are photostability studies**.** Photostability of API depends on the factors connected with the irradiation features (wavelength, intensity and time of exposure) as well as on a variety of physico-chemical features characterising the API substance itself (formulation type: solution, powder, crystal; presence of specific molecular fragments). Although a large number of drugs have the form of solid formulations, most studies on photostability have been performed in artificial conditions, i.e. in dilute solutions in specific organic solvents in which such processes can be easily

observed. However, it should be mentioned that in such conditions the drug's photostability may be totally different than in solid. Even the addition of excipients may affect the photostability of drug formulation.

The main phenomenon responsible for deterioration of the useful properties of a drug, that is the loss of its activity or increased toxicity, is photodegradation. Photodegradation of a drug is significant under irradiation with light of wavelength longer than 330 nm. However, even in such favourable conditions, photoreaction can be easily unnoticed when it is very slow, especially in solid state. The typical approach to study photodegradation is based on a combination of different techniques. The widely applied procedure includes studies of photodegradation products using different techniques including chromatographic techniques gas/liquid chromatography (GLC), thin layer/chromatography (TLC) or high pressure liquid chromatography (HPLC), electron paramagnetic resonance (EPR), differential thermal analysis (DTA), nuclear magnetic resonance (NMR), and *Fourier transform infrared* reflection absorption spectroscopy (FT-IR RAS). Although the above mentioned methods are very sensitive but they require the use of solvents which may cause further degradation of drug during the analysis. The alternative for the identification of photodegradation products are solid state methods, which allow credible identification of the compounds in solid, guaranteeing elimination of the above mentioned solvent side effects.

An interesting example illustrating the studies of photostability is nifedipine, mainly used for treatment of hypertension, coronary heart/artery diseases and arrhythmias, or as cardiovascular organ-protective agent [176]. Nifedipine is known as one of the most photolabile APIs, extremely sensitive to UV-VIS radiation up to 450 nm and to the ionising radiation. The loss of therapeutic properties by NIF due to photodegradation is well documented [177]. The photodegradation of NIF in solution has been widely studied [178] whereas only a few authors have studied it in solid [179]. The photostability and the photostabilization of NIF in different solid formulations including powders and tablets have received considerable attention [180]. A typical approach to study these phenomena is based on a combination of different techniques. The products of photodegradation are studied using different techniques including EPR, DTA, NMR, FT-IR-RAS but the isolation of the products is performed by chromatographic techniques: gas/liquid chromatography (GLC), thin layer/chromatography (TLC) or high pressure liquid chromatography (HPLC).

Unfortunately, solid ^{13}C NMR is not always able to detect the photoproducts. A striking example is nifedipine whose degradation effect manifested as shifts of signals assigned to C(4), C(3) and C(5) as well as C(2') sites was observed only in solution [181]. Instead, a combination of ^{14}N NQDR and DFT methods proved to be effective because the nitrogen sites in nifedipine and its photoproduct differ significantly, (-NH- versus -N= and -NO$_2$ versus -NO). ^{14}N NQDR seems the best experimental method of choice for non-destructive detection of NIF to NO-NIF photoconversion in solid [182]. It is worth noting that the electron density distribution at the -N=, -NH, -NO$_2$, -NO sites can be accurately and fully experimentally evaluated using this technique. Thus ^{14}N NQDR should be of particular importance for the studies of photodegradation of solid drugs, especially nitro-compounds. Additionally within QTAIM formalism it has been possible to predict the sites in nifedipine most affected by irradiation -NH, -NO$_2$ and -CH.

It is known that NQR is suitable for determination of the degree of ^{35}Cl release from a given pharmaceutical under the effect of UV irradiation, which is manifested as a decrease in the signal intensity or even as its disappearance (e.g. for furosemide), but when the radicals

formed undergo fast recombination the relevant changes in the NQR spectra are undetectable (e.g. thiazides).

4. Conclusions

According to James Black, a Nobel laureate in Physiology or Medicine 1988, '*The most fruitful basis for the discovery of a new drug is to start with an old drug.*' [183]. Actually among 700,000 crystal structures from monatomic metals to proteins and viruses systematically collected from the late 1960s and early 1970s are available in five comprehensive and fully retrospective world depositories: Cambridge Structural Database (CSD), Worldwide Protein Data Bank (wPDB), Nucleic acid database (NDB), Metals and intermetalics database (CRYSTMET) and Inorganic Crystal Structure Database (ICSD) only about 10% are structures of APIs and targets. The largest database, CSD, nowadays contains over 500,000 structures ("small molecules" - less than 1000 atoms) of which 99% were determined by XRD [61,62]. The number of drug-like structures in CSD exceeds 60,000. The largest protein archive, wPDB, contains 73,699 experimentally-determined structures of proteins, nucleic acids, and complex assemblies, of which 87% were determined by XRD, 12% by NMR, less than 1% by electron microscopy and hybrid methods.

Thus among the large number of known, classified drugs and targets only a small fraction has been investigated using SS NMR and even a smaller one - using NQR. In fact the application of both methods in drug development studies has definitely a short history. Despite this, significant improvements in the past few years, resulting in a remarkable enhancement of the speed and the efficacy of this approach permit expecting a great increase in its role in the near future. SS NMR and NQR in combination with computational methods like ab initio or DFT will play a crucial role in modern crystallography and medicinal chemistry. An important advantage of SS NMR or NQR spectroscopy over XRD, is that SS NMR and NQR spectroscopies are sensitive to local or short-range order (<5 Å), while XRD detects comparatively long-range order (>100 Å). Thus SS NMR and especially NQR provides unique insight into intermolecular interactions that are not easily obtained by other methods. A combination of experimental and theoretical techniques has certainly much to offer for the studies of drugs or potential drugs in early phases of drug research. Their capabilities in particular when applied to the known drugs, have been well documented in literature [92,99,101,104,105,148,156-161,184-189].

Although in some fields the NMR or NQR are not competitive to IR, UV or XRD the examples of combined studies given above show that both techniques have a potential to elicit structural and physico-chemical information about a molecule of dug, target or the interaction drug-target.

5. References

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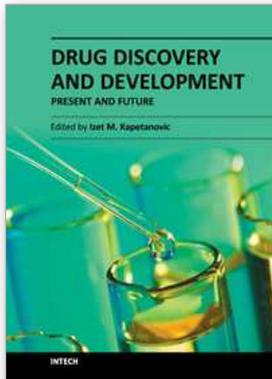
Drug Discovery and Development - Present and Future

Edited by Dr. Izet Kapetanović

Drug discovery and development process aims to make available medications that are safe and effective in improving the length and quality of life and relieving pain and suffering. However, the process is very complex, time consuming, resource intensive, requiring multi-disciplinary expertise and innovative approaches. There is a growing urgency to identify and develop more effective, efficient, and expedient ways to bring safe and effective products to the market. The drug discovery and development process relies on the utilization of relevant and robust tools, methods, models, and validated biomarkers that are predictive of clinical effects in terms of diagnosis, prevention, therapy, and prognosis. There is a growing emphasis on translational research, a bidirectional bench to the bedside approach, in an effort to improve the process efficiency and the need for further innovations. The authors in the book discuss the current and evolving state of drug discovery and development.

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