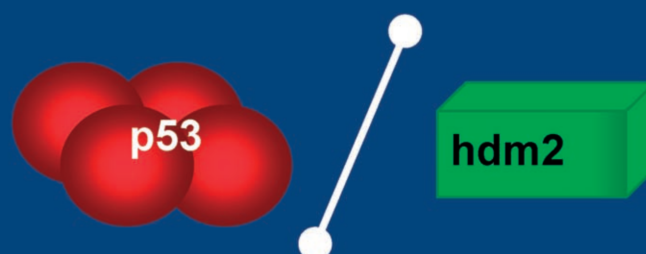
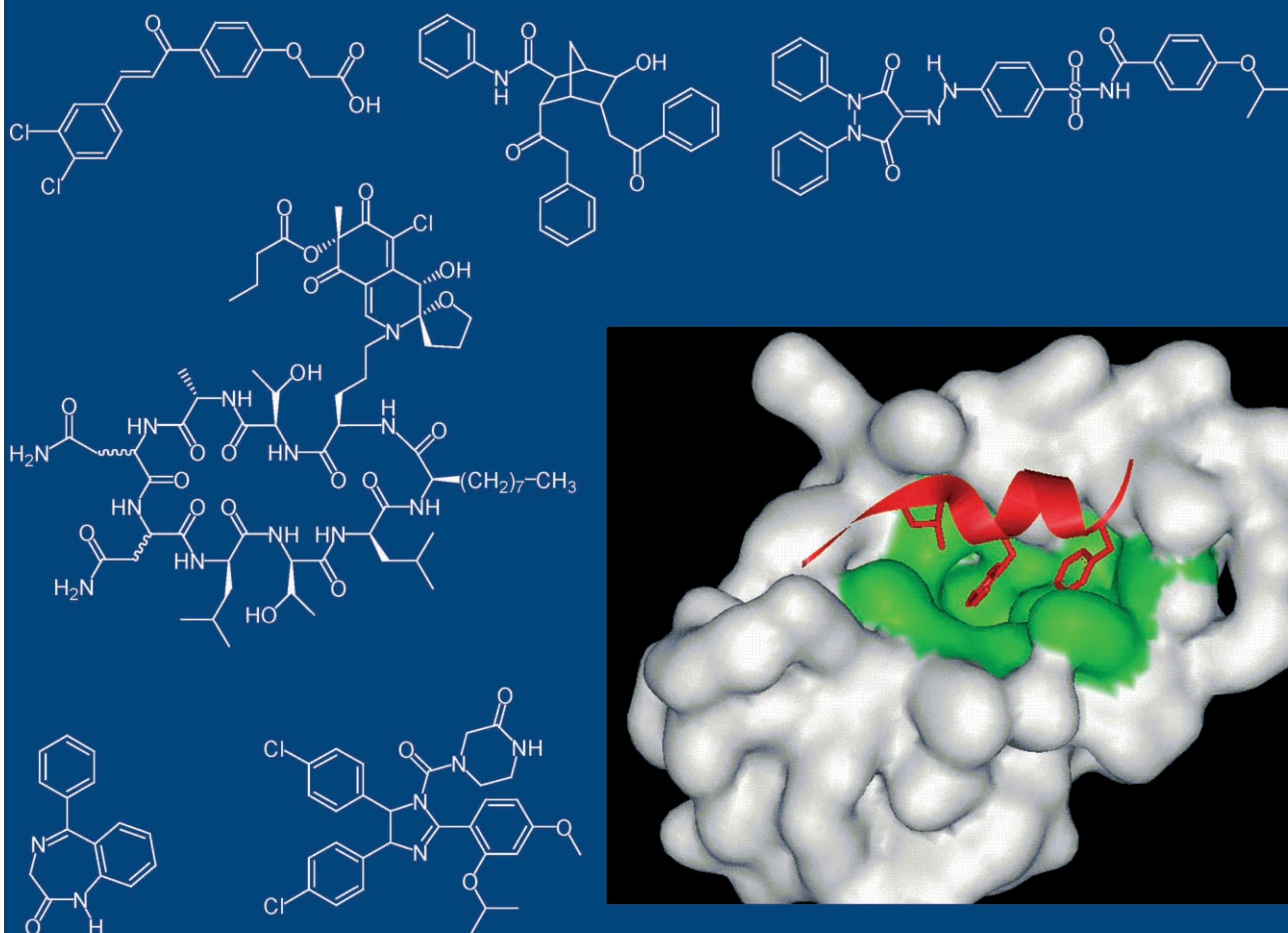
Blocking Protein–Protein Interactions





 DOI: 10.1002/cmdc.200600004 © 2006 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim

DOI: 10.1002/cmdc.200600004

Drugs Targeting Protein–Protein Interactions**

Patrick Chène*[a]

Most biological processes involve permanent and nonpermanent interactions between different proteins, and many protein complexes play a key role in various human diseases. Therefore, molecules that prevent the formation of these protein complexes could be valuable new therapeutic agents to treat these diseases. Protein interfaces have not evolved to bind low-molecular-weight molecules, as is the case with enzyme catalytic sites. It is therefore difficult to identify small compounds that inhibit protein–protein interactions. However, there is considerable diversity in the structure of protein interfaces, some of which may be more attractive than others for medicinal chemistry. One of the main challenges in drug discovery is to identify these interfaces and to exploit their properties to make marketable drugs. Herein, the properties of protein interfaces are discussed in light of their use as drug targets.

1. Introduction

The discovery of new drug targets is a constant challenge for pharmaceutical companies. Over the last few decades, most drugs that have been developed are enzyme inhibitors.[1] One explanation for this preference is that enzymes bind naturally to small molecules: their substrate. This offers the possibility of identifying small molecules that mimic the substrate and bind to these proteins, thus inhibiting their biological activity. For example, transition-state analogues bind with high affinity to enzymes and are potent inhibitors.[2] Furthermore, as enzyme inhibitors are normally small molecules, they usually have an acceptable bioavailability, which facilitates their development. Today, however, while many enzymes have still not been targeted or are in the process of being evaluated in a more systematic fashion,[3] the pharmaceutical industry is looking for new opportunities outside the enzyme field. Amongst the potential candidates, inhibitors of protein–protein interactions represent an attractive new class of molecules. Many proteins, including enzymes, exert part if not all of their biological activity by interacting with other proteins. The prevention of these interactions is a way of modulating the activity of such proteins.

The structural diversity and large number of protein–protein interfaces offer an enormous amount of new targets for the pharmaceutical industry. Certain caution is required, however, because the number of possible new targets may not be as large as it appears. Protein interfaces have not evolved to bind to low-molecular-weight molecules, as have enzymes. It may therefore be more difficult to identify inhibitors of protein–protein interactions than it is to identify enzyme inhibitors. A second difficulty comes from the diversity of protein interfaces. As large families of enzymes bind to the same substrate (for example, ATP for the kinases), it is possible to use the knowledge gained and the compound libraries that were synthesized to target the first members of the family to more rapidly design compounds that target new members of the family. This, of course, dramatically enhances the speed of the drug-discovery process. In the case of protein–protein interactions, even if similarities are observed between some interfaces, it does not appear that binding sites are preserved amongst protein interfaces. Therefore, each protein interface is rather unique, and new strategies in chemistry (synthesis and optimization of new scaffolds) may have to be developed for each new protein–protein interaction studied. This is, of course, more time-consuming and less attractive for pharmaceutical companies because they have to maintain high productivity in the very competitive field of drug discovery. The following presents an overview of the properties of protein interfaces followed by the application of this knowledge to the design of competitive inhibitors of protein–protein interaction.

2. The Diversity of Protein–Protein Interfaces

In living organisms, a large number of proteins form transient or permanent complexes to exert their biological function, and recent studies have revealed the complexity of these protein–protein interaction networks.[4] As so many protein–protein interactions occur in cells, one can expect differences in the structure and composition of the regions of the proteins committed to the formation of these complexes. These differences are necessary to reach the degree of specificity needed to form the "right" complexes in the crowded cellular environment and to obtain complexes with different stabilities. For example, the protein concentration in the endoplasmic reticulum is estimated to be 100 $mg\,mL^{-1}$.[5] This diversity of the protein

[a] *P. Chène*
Oncology Research
Novartis Institutes for Biomedical Research
4002 Basel (Switzerland)
Fax: (+41) 616-963-835
E-mail: patrick_chene@yahoo.com

[**] *This review will also appear as a chapter in the book* Chemical Biology—From Small Molecules to Systems Biology and Drug Design *(Eds.: S. Schreiber, T. Kapoor, G. Wess), Wiley-VCH, Weinheim, which is scheduled for publication in October 2006.*

 © 2006 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim

interfaces is an opportunity for drug discovery because it may allow more specific inhibitors to be generated. However, it is very likely that many of these interfaces do not have the properties required for the design of potent inhibitors. It is therefore very important—before starting any drug-discovery process aimed at the design of competitive inhibitors of protein–protein interactions—that the druggability of the selected interfaces be evaluated. This depends on both the structure and the physicochemical properties of the interface. In this section, the general properties of protein interfaces are summarized.

Protein complexes are formed from identical subunits (homo-oligomers) or from different subunits (hetero-oligomers).[6] These oligomers can be formed directly during protein synthesis (obligate complexes) or on the encounter (non-obligate complexes) between different subunits. Protein complexes also have various half-lives. Permanent complexes are very stable, and their subunits remain associated, whereas others exist only transiently (non-permanent) and their chains associate and dissociate more easily. This means that the subunits of some protein complexes (obligate/permanent) never exist in cells as stable independent structures. Furthermore, the formation of some complexes depends on the presence of effector molecules such as GTP, changes in protein expression and localization, or physiological conditions such as pH.

These general properties are already valuable for drug discovery. Targeting the interface of permanent oligomers a priori is a difficult task, because the only way to abolish this type of interaction is to identify compounds that act during protein synthesis and folding. However, it is conceivable that compounds may be identified which, upon binding to the contact surface of one subunit, prevent interaction with the other subunit in nonpermanent oligomers. Finally, the synthesis of compounds that mimic the natural effector might be an attractive way of inhibiting the formation of effector-regulated complexes. In this case, the inhibitors are designed in such a way that they bind not at the protein interface, but to the effector-binding pocket. Depending on the structure of the effector-binding site, the design of such inhibitors might be similar to the design of enzyme inhibitors. This type of approach is not considered herein, as this Review focuses on compounds which, upon binding at protein interfaces, prevent the association between two proteins (competitive inhibitors).

Upon binding, the components of a protein complex bury part of their accessible surface to create the contact interface. On average, the size of the subunit interface in permanent homodimers is larger than in other protein complexes.[6,7] Jones and Thornton studied a set of 59 complexes and found that the surface buried in homodimers varies from 368 Å^2 to 4746 Å^2, whereas in heterocomplexes, it ranges from 639 Å^2 to 3228 Å^2.[8] Janin and collaborators also found similar results.[7,9] The study of the structure of the free and associated subunits shows that they are likely to undergo conformational changes when they form large interfaces ($>$1500 Å^2).[10,11] With the exception of co-expressed subunits (obligate complexes),[11] it does not seem that there is a strong correlation between the size of the interface and the binding energy (ΔG^1).[12] However, the entire contact surface does not contribute equally to binding. Some regions—recognition patches or hot spots—are more important for recognition and binding.[13] These regions have a core and a rim[14] with the more accessible rim residues surrounding the more buried core residues. The amino acid composition of the rim is similar to the rest of the protein surface, whereas the core contains more aromatic residues which imparts a higher lipophilicity to this part of the contact region. There is a correlation between the number of recognition patches and the size of the interface.[7,14] The larger the interface is, the more hot spots are present. However, in most cases, only one hot spot is present at the interface, and on average it buries a surface area of 1560 $\pm$ 340 Å^2 upon binding. For interfaces with multiple recognition patches, one patch is generally larger and has a size similar to the one of the hot spots found in single-patch interfaces. The presence of recognition patches at protein interfaces is interesting for drug discovery. Compounds that interact with these hot spots should prevent interaction because a large part of the binding energy is concentrated in these areas. As the hot spots are smaller than the full interface, it might be easier to identify low-molecular-weight compounds—molecules similar in size to enzyme inhibitors—that inhibit interaction. In contrast, if the binding energy were equally distributed over the entire interface, much larger molecules with a lower likelihood of success as drug-development candidates would have to be designed.

The shape of the interface is another important parameter for drug discovery because it is more difficult to obtain potent inhibitors for flat interfaces than for interfaces which contain well-defined cavities (pockets). The less flat the interface between two proteins, the greater the tendency of one partner to be buried and to form a more stable complex. The heterocomplexes have more planar interfaces than homodimers, and permanent heterocomplexes have more twisted contact surfa-

Patrick Chène received his chemical engineering diploma at the Ecole Normale Supérieure de Chimie in Toulouse, France. He completed his PhD in molecular pharmacology at the Laboratoire de Pharmacologie et de Toxicologie Fondamentales du CNRS, France. After a postdoctoral position in enzymology in the research group of Professor Alan Fersht at Cambridge University, England, he joined Ciba–Geigy in Switzerland, where he worked on the determination of the structure of protein kinases. At the Dana–Farber Cancer Institute, USA, and Novartis, Switzerland, he guided research activities aimed toward the identification of inhibitors of the p53–hdm2 interaction. He was later in charge of drug-discovery programs in the ATPase field. He is now leading the Druggability Enzymology Profiling group within the Oncology Department at Novartis in Basel.

[1] *The change in Gibbs free energy (ΔG) is linked to change in enthalpy (ΔH) and entropy (ΔS) by the following formula: $\Delta G = \Delta H - T\Delta S$. A process occurs spontaneously at constant temperature and pressure if $\Delta G < 0$.*

 © 2006 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim

ces than those that are nonpermanent.[8] This suggests that the most attractive complexes for drug discovery—the nonpermanent complexes (see above)—have rather flat interfaces. The presence of cavities or pockets at the contact region should therefore be looked at very carefully during the evaluation of a protein–protein interaction.

Even if cavities are present at an interface, they must be suitable for drug discovery. Of course, they must be large enough to accommodate inhibitors, but their shape complementarity is also important. It might be more difficult to generate potent competitive inhibitors if the two interacting chains are closely packed and make an extensive number of direct interactions.[2] In contrast, if the shape complementarity within the cavity between the two chains is low, the interacting subunits may only make a limited number of direct interactions. For such cavities, it might be easier to improve the potency of the inhibitors. A potent inhibitor should contain chemical groups, which, upon binding to the target protein, mimic the key interactions (the most important for ΔG) made by the competing subunit and chemical groups, which make new interactions with the target protein. The creation of these additional contacts between the inhibitor and the target protein leads to a favorable enthalpic contribution ($\Delta H < 0$) in the binding energy and therefore to an increased potency. "Loose" interfaces have a higher probability of containing atoms not directly involved in the formation of the protein complex than do very complementary protein contact regions. They therefore offer more possibilities for improving the potency of inhibitors. Several methods are used to determine the complementarity between two interacting proteins.[10] Thornton and collaborators[8] used one method, the gap index, to measure the complementarity of different complexes. Their results show that the homodimers and permanent heterodimers make more complementary interfaces than the non-obligatory heterocomplexes. The latter may therefore be more "druggable". It should be kept in mind that these methods give an indication of the atom density (packing) but not of the interaction network. As it is important for the enhancement of potency that inhibitors make more interactions than the competing chain, loose packing does not necessarily imply that the cavity is a good drug target. During the study of an interface, therefore, it is important, even in the case of "loose" interfaces, to carefully check that in addition to the key interactions made in the protein complex, it is possible to create new interactions that will help to enhance the potency of the inhibitors.

One consequence of a lack of complementarity between two interacting proteins is that water molecules are present at the interface to satisfy the H-bond network between the subunits. The study of different protein interfaces shows that contact regions with few cavities do not contain many water molecules, whereas interfaces with more cavities contain a larger number of water molecules that are used to maintain close packing at the interface.[15] These trapped water molecules are involved in bridging H bonds between the two chains.[15] Water is therefore an important element of the interaction, and it should be considered during drug design. The displacement of key bound water molecules by the inhibitor might enhance its affinity because of a favorable entropic effect.

The presence of water molecules at the interface reflects its polar nature, but protein contact regions also contain hydrophobic areas which are important for the interaction. In terms of energy, hydrophobic interfaces are more suitable for drug discovery than polar regions. The partial desolvation of both the protein and the inhibitor upon binding is a favorable component of the binding energy. The design of molecules that contain lipophilic moieties is thus a prerequisite to obtaining potent drugs. The chemical nature of protein interfaces has been extensively studied, and their content of polar/nonpolar groups has been analyzed.[6–10,14,16–18] On average, protein interfaces are composed of 56% nonpolar carbon-containing groups, 29% neutral polar groups, and 15% charged groups.[10] The interfaces in permanent complexes are generally more hydrophobic than the those of nonpermanent complexes.[6] This could be explained by the fact that solvent-exposed hydrophobic patches are energetically unfavorable and that subunits with hydrophobic surfaces are therefore not stable. The presence of hydrophobic cavities at the interface between two proteins is particularly attractive for drug discovery because it allows the design of lipophilic molecules which, upon binding, become buried in a hydrophobic environment.

Another feature of the interaction between two proteins is the loss of flexibility of their contact regions upon binding. It is expected from thermodynamics that better binding is obtained if the interaction does not induce a large loss of conformational entropy. Indeed, it has been shown that protein interaction sites are less flexible than the rest of the protein surface.[19] The loss of flexibility that occurs during the association between two proteins can be advantageously used to design inhibitors. The design of compounds conformationally constrained in such a way that they already take on their bound conformation in solution is a way of improving potency. Such molecules will not undergo large conformational changes upon binding, and they will therefore "pay" a decreased entropic penalty in comparison with more flexible inhibitors.

Altogether, this short summary indicates that there is no common recognition template used by oligomeric proteins to form complexes. In contrast, even if protein interfaces share some general properties, they differ to a large extent. It is therefore very difficult to make a general statement on the druggability or non-druggability of protein interfaces. Among the large number of protein interfaces, some are more druggable, and a major challenge for drug discovery is to identify them.

3. A Proposed Decision Tree to Select Interfaces for Drug Discovery

To help in the identification of druggable interfaces, a decision tree is proposed. Two points need to be addressed before describing this tree. First, drug discovery is not, at least today, an exact science. Even if an interface does not fit the decision

[2] A direct interaction is an interaction that does not involve any bridging water molecules between the two interacting protein subunits.

 © 2006 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim

tree, it might still be possible to obtain molecules that prevent its formation. This leads to the second point: the potency of protein–protein interaction inhibitors. In many cases, molecules (peptides or low-molecular-weight compounds) with IC_{50}[3] values in the micromolar range are described as inhibitors of protein–protein interactions. Although they may be useful tools to study the interaction, a large number of these compounds will never enter clinical use, which is the ultimate goal for pharmaceutical companies. These molecules need further optimization to reach this goal. Protein–protein interaction inhibitors will only be considered attractive new drugs when they can demonstrate clinical efficacy, as do enzyme inhibitors. Such drugs can only be obtained if the target interface allows the design of potent and bioavailable molecules. A detailed analysis of the interface to assess its druggability is therefore required before any drug discovery program can be started. The proposed decision tree may help in the selection of the interfaces which possess the structural and physicochemical properties required for the design of potent inhibitors (Figure 1).

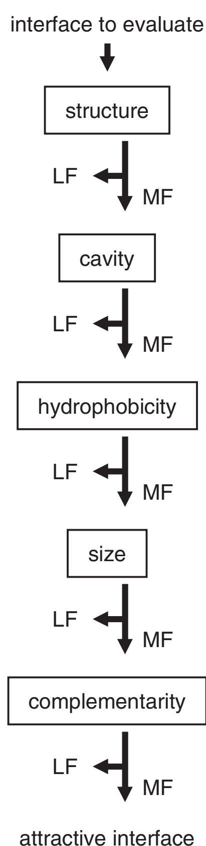


Figure 1. A decision tree to evaluate the druggability of protein interfaces. This tree can be used to determine whether a selected interface possesses some of the features required for drug discovery. LF: less favorable; MF: more favorable.

Just as it is easier to pick cherries from a cherry tree in daylight than during a moonless night, so too is it easier to guide the drug-discovery process if it is possible to see the structure of the target interface. A drug-discovery program can also be successful without the use of structural information, but it might be harder and take longer to obtain potent molecules without this precious knowledge. The structure of the interface should help in deciding whether it is druggable by using the criteria described below but it will also help to improve the potency of the compounds during their optimization. This explains why the availability of the interface structure is considered the most favorable case in the decision tree.

The second criterion in the decision tree is the presence of cavities at the interface. The most favorable case is when a well-defined binding pocket is found at the contact region between both proteins. The presence of such a pocket allows the formation of a stable inhibitor–protein complex if the inhibitor mimics the protruding chain. The contact region of some non-complexed proteins is flexible, and upon binding, this plasticity/flexibility allows conformational changes that enhance interface complementarity. The structure of the final protein complex may therefore not reveal the presence of cavities which are present on the surface of the unbound proteins but which are absent in the final complex. Compounds that bind to these pockets could block the conformational changes required for the formation of the complex, preventing the interaction. The knowledge of the structure of the unbound proteins is therefore very useful in the identification of this type of pocket.

The next selection criterion concerns the polarity of the selected cavity. In the most favorable case, it should contain hydrophobic residues to favor the design of lipophilic inhibitors. The addition of hydrophobic substitutions (taking care to keep good solubility) is an effective way to improve the potency of an inhibitor owing to the hydrophobic effect. It has been shown that electrostatic interactions are important for the rate of association, but not for the stability of protein complexes.[20] Furthermore, electrostatic interactions are weakened by the high dielectric constant of water. It might therefore be more difficult to identify inhibitors that bind tightly to the target cavity if it is essentially polar.

The presence of a hydrophobic cavity is important, but its size is also relevant for drug discovery. It should be large enough to accommodate an inhibitor. An analysis of 20 marketed drugs shows that they have a solvent-accessible surface that ranges from 150 to 500 $Å^2$,[21] so the target cavity should accommodate such molecules. On the other hand, the cavity should not be so large that the key contact residues for the interaction are too distant from each other. In such cases, inhibitors designed to contact these different residues might be excessively large. Keeping the size of inhibitors small is important for their bioavailability. As a general trend, the larger a synthetic molecule is, the lower its bioavailability.

The last criterion of the decision tree is the shape complementarity between the two interacting subunits within the cavity. The less favorable case is that in which both chains are densely packed and make many direct interactions within the cavity. As already mentioned, inhibitors should mimic the natural substrate, but they should also make additional contacts that help to enhance their potency. The cavity should therefore contain atoms that are not directly engaged in the interaction between both proteins such that it is possible to design molecules which interact directly with them. Interfaces that possess cavities with low complementarity might then be more attractive. As water molecules are present in such cavities, the potency of the inhibitors could be enhanced if they are designed in such a way that upon binding they displace some key water molecules.

The analysis of protein interfaces with the proposed decision tree leads to the selection of competitive inhibitors because it focuses on the characterization of the contact region between both proteins. However, it is important to note that molecules that do not bind at the interface can also inhibit protein–protein interactions. The potency of competitive inhibitors, as determined by the measure of their IC_{50} value, is affected by the concentration of the substrate (Figure 2). The higher the con-

[3] *IC_{50}: concentration of inhibitor required to block 50% of the interaction between two proteins.*

 © 2006 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim

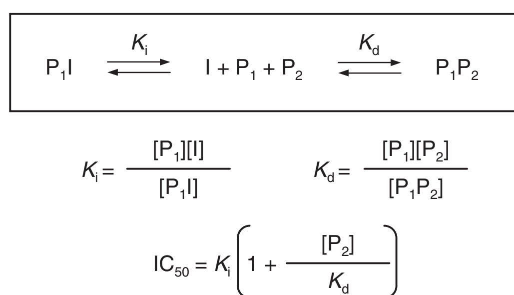


Figure 2. Competitive inhibition: the inhibitor (I) binds to the target protein P_1 blocking its association with protein P_2. IC_{50} corresponds to the concentration of inhibitor required to inhibit/inactivate the P_1P_2 complex by 50%. Note the influence of $[P_2]$ on IC_{50}. Cheng and Prusoff have published a detailed analysis on the relationship between IC_{50} and inhibition of enzymes.[60]

centration of the substrate is, the less potent the inhibitor becomes. Therefore, if the competing subunit is very abundant and/or very stable (low turnover) so that it accumulates after inhibition of the interaction, it might be more difficult to reach efficacy with low doses of a competitive inhibitor. Higher doses of inhibitor would have to be administered to counterbalance this effect, but then compound-related toxicity could arise. Molecules that are not competitive inhibitors do not suffer these disadvantages. These molecules, which do not bind at the interface, induce conformational changes that prevent complex formation. Several such allosteric inhibitors have been identified; for example, see Arkin, M. R. in Table 1. However, it is very likely that it this strategy does not apply to every protein complex. Furthermore, if such binding sites do exist, they must also possess structural and physicochemical properties which allow the design of potent compounds.

4. Experimental Validation of the Selected Interface

All the selection criteria presented in the decision tree in Figure 1 are general, and many protein interfaces will only fulfill some of them. In these cases, as well as those for interfaces that meet all the decision-tree criteria, an experimental study of the interface should be carried out before drug-discovery activities are started. This experimental validation should enable a good level of confidence to be obtained on the druggability of the selected interface.

A powerful way of performing this experimental validation is to combine site-directed mutagenesis and peptide-binding experiments. Site-directed mutagenesis is used to demonstrate the role of selected residues in the interaction, while peptides assist in mapping the binding site and also in defining the importance of key amino acids. The synthesis of peptides containing nonnatural amino acids can also be used to create new contacts with the targeted subunit. This should help in validating some optimization strategies that could be used later on in the design of low-molecular-weight compounds. It must be kept in mind that peptides can only be used if at least one of the two contact regions at the interface is formed by a contiguous stretch of amino acids. This is not often the case, and many protein-binding sites are fragmented.[8]

Peptides are also useful tools to demonstrate the validity of the biological concept and thereby show that the inhibition of the selected protein–protein interaction leads to the expected phenotype. As peptides generally have a low bioavailability, they often have to be coupled to special sequences that facilitate their transport into cells.[22] Finally, the peptides can serve as starting points for a drug-discovery program. They can be transformed into peptidomimetics which, in some cases, can be further "depeptidized".

Table 1. Selected review articles that cover the latest findings in the discovery of inhibitors of protein–protein interactions.

First Author	Title	Reference[a]
M. R. Arkin	Small-molecule inhibitors of protein–protein interactions: progress towards the dream	*Nat. Rev. Drug. Discovery* **2004**, *3*, 301.
L. Pagliaro	Emerging classes of protein–protein interaction inhibitors and new tools for their development	*Curr. Opin. Chem. Biol.* **2004,** *8*, 442.
Y. L. Janin	Peptides with anticancer use or potential	*Amino Acids* **2003,** *25*, 1.
T. Berg	Modulation of protein–protein interactions with small organic molecules	*Angew. Chem.* **2003,** *115*, 2566; *Angew. Chem. Int. Ed.* **2003,** *42*, 2462.
A. Loregian	Protein–protein interactions as targets for antiviral chemotherapy	*Rev. Med. Virol.* **2002**, *12*, 239.
D. A. Ockey	Inhibitors of protein–protein interactions	*Expert Opin. Ther. Pat.* **2002**, *12*, 393.
Z. Huang	The chemical biology of apoptosis: exploring protein–protein interactions and the life and death of cells with small molecules	*Chem. Biol.* **2002**, *9*, 1059.
R. Pérez-Montfort	The interfaces of oligomeric proteins as targets for drug design against enzymes from parasites	*Curr. Top. Med. Chem.* **2002,** *2*, 457.
P. L. Toogood	Inhibition of protein–protein association by small molecules: approaches and progress	*J. Med. Chem.* **2002**, *45*, 1543.
A. G. Cochran	Antagonists of protein–protein interactions	*Chem. Biol.* **2000**, *7*, R85.
J. Zeng	Computational structure-based design of inhibitors that target protein surfaces	*Comb. Chem. High Throughput Screening* **2000**, *3*, 355.
Z. Huang	Structural chemistry and therapeutic intervention of protein–protein interactions in immune response, human immunodeficiency virus entry, and apoptosis	*Pharmacol. Ther.* **2000**, *86*, 201.

[a] These articles cover the period 2000–2004.

 © 2006 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim

5. Screening Techniques, Compound Libraries, and Targets

As the goal of any drug-discovery program that deals with a protein–protein interaction is to identify low-molecular-weight compounds that bind to a well-defined pocket, the technologies and compound libraries used to identify enzyme inhibitors can also be used to identify inhibitors of protein–protein interactions.

Various assays are used to identify competitive inhibitors of protein–protein interactions, but those with which the inhibition of the complex is directly measured—competition assays—are the most commonly used. Several assay formats are available: ELISA, fluorescence polarization, fluorescence resonance energy transfer, and others. These assays are designed in such a way that they use either the two full-length proteins, only their interacting domains, or when possible, even peptides that mimic the binding region. One must be very cautious with this type of assay for the determination of IC_{50} values. The potency of competitive inhibitors depends on the amount of the competing protein present in the assay (Figure 2). The amount of competing protein present in the assay may vary between laboratories and even between different protein batches (change in specific activity). To obtain an accurate estimate of the binding properties of the inhibitors, their K_d[4] values should be measured. The data obtained with the competition assay should therefore be completed with the K_d measurements obtained, for example, by isothermal calorimetry. Calorimetric measurements also provide valuable information about the energy of the interaction, which can be used to further optimize the compounds (for example, to generate more enthalpy-driven or entropy-driven compounds[23]).

The other assays used to identify inhibitors of protein–protein interactions are binding assays. In these cases, only one of the two interacting chains is present, and the binding of the compounds to this protein is measured. Several assay formats are used: surface plasmon resonance, ^{1}H–^{15}N HSQC NMR spectroscopy, ultracentrifugation, and others. Many of these methods only indicate that the compounds bind to the target protein, but they do not show that such binding inhibits the interaction. This needs to be demonstrated in a subsequent analysis such as a competition assay.

It is important to note that in some competition and binding assays, it is difficult to directly determine whether the inhibiting molecules are competitive inhibitors or not. The inhibitors may bind to a pocket located outside the interacting region and modulate the interaction by an allosteric effect. To allow a better optimization of these inhibitors, their binding mode should be firmly demonstrated. It is essential in this process to determine the structure of the inhibitor–protein complex.

All types of compound libraries can be screened to identify protein–protein interaction inhibitors: low-molecular-weight compound libraries, natural compound libraries, peptide/peptidomimetic libraries, combinatorial chemistry libraries, fragment libraries and so on. A simple survey of the literature shows that molecules belonging to these different types of libraries are described as protein–protein interaction inhibitors. However, there is an argument that is sometimes raised in the literature regarding the diversity of compounds in these libraries: the libraries available in pharmaceutical companies reflect their drug-discovery history. As most companies have focused on the design of enzyme inhibitors, it is possible that the structural diversity of their libraries might not match what is required to identify inhibitors of protein–protein interactions. Although this might be the case, the increasing number of drug-discovery programs that deal with protein interfaces will ensure that the chemical diversity of these libraries will change, and they may contain more compounds that prevent protein–protein interactions. An alternative explanation for the low success rate with random screens of large libraries for inhibitors of protein–protein interactions is that the selected interfaces have low druggability and that independent of the chemical diversity of these libraries, the probability of finding inhibitors is also low.

The availability of the three-dimensional structure of the protein complex allows structure-driven drug-discovery approaches. In this case, a pharmacophore model is first established. This corresponds to identifying the interactions that take place at the interface and that contribute most to ΔG. The importance of these interactions can be validated by site-directed mutagenesis or, if possible, by the use of peptides. Once these interactions are validated, molecules containing chemical groups that mimic these key interactions are selected from compound libraries and tested. Very often these initial molecules are not optimal (for example, they do not make all the key contacts) and they must be modified to enhance their potency. This is done, for example, by adding the missing pharmacophores and/or by creating contacts which are not present in the natural complex. Alternatively, de novo drug design may be carried out. In this case, a "very basic" scaffold, which mimics only a few of the key interactions made by the competing subunit, is selected and modified progressively to obtain molecules that contain the different pharmacophores. This, of course, is very time-consuming and resource-demanding, because the affinity of the initial scaffold is usually low, and a great deal of chemistry is required to improve its potency. The structure-driven and screening approaches are not mutually exclusive, but the former require good comprehension of the interaction, while the latter can be used without information regarding the target interface.

The list of protein–protein interactions that have been the subject of drug-discovery programs is constantly growing, and many excellent articles have reviewed the latest findings in this area. Some of these reviews are listed in Table 1, and can provide the reader with an idea of the protein–protein interactions that have already been selected as targets for drug-discovery programs and on the inhibitors that have been identified in these studies. In the following, we focus on one protein interface: that of the p53–hdm2 interaction. This protein–protein interaction has been selected from the literature because, with the various results put together, the work carried out by the different research groups working on this interface makes

[4] K_d: *apparent dissociation constant of the protein–inhibitor complex.*

 © 2006 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim

up a very nice case study for the design of competitive inhibitors of protein–protein interactions.

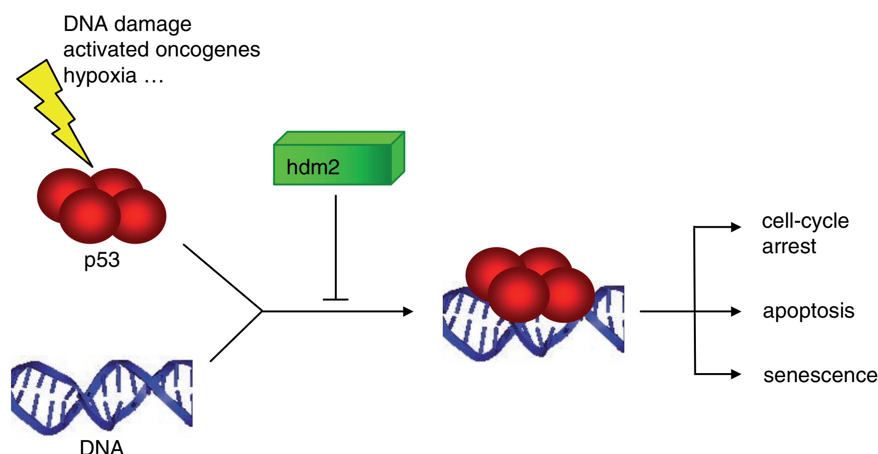


Figure 3. Regulation of p53 by hdm2: the tumor suppressor p53 is a tetrameric transcription factor. Upon various stress conditions such as DNA damage, activation of various oncogenes, or hypoxia, p53 is activated and binds to DNA. Depending on the cell line and/or the nature of the cellular stress, p53 induces either a cell-cycle arrest or apoptosis. p53 is also able to mediate other biological answers such as senescence. hdm2 is a negative regulator of p53; upon binding to p53 it inhibits its transcriptional activity, promotes its degradation, and favors its export from the nucleus. Therefore, in the presence of hdm2, the tumor-suppressor activity of p53 is inhibited.

6. An Example: the Design of Inhibitors of the p53–hdm2 Interaction

Biological background

The p53 protein is a transcription factor that regulates the expression of several genes with different biological functions, such as cell-cycle regulation, apoptosis, DNA repair, and differentiation.[24] The loss of p53 function has dramatic consequences, and the *p53* gene is deleted or mutated in more than 50% of human cancers.[25] The overexpression of the hdm2 protein can also lead to the inactivation of p53. The p53 and hdm2 proteins form an autoregulatory feedback loop:[26,27] p53 stimulates the expression of hdm2, which in turn acts negatively on p53 in several ways (Figure 3). It inhibits its transcriptional activity,[28] promotes its degradation,[29,30] and favors its export from the nucleus.[31] The *hdm2* gene is amplified in about 7% of human cancers,[32] and hdm2 is overexpressed in different tumor types.[33,34] It is therefore likely that the p53 pathway is not active in these tumors, because the overexpressed hdm2 protein constantly inhibits the p53 protein. The idea that has been pursued by several pharmaceutical companies is the generation of molecules which, by preventing the p53–hdm2 interaction, will activate the p53 pathway in such tumors and thereby show anticancer activity.

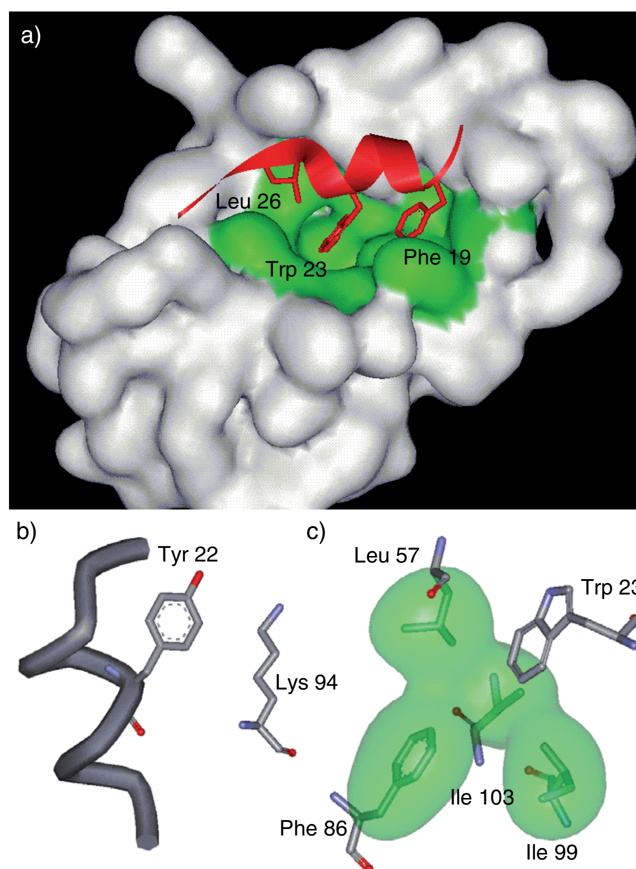


Figure 4. The structure of p53 (residues 17–29) in complex with hdm2 (residues 25–109).[39] a) The surface of hdm2 is represented in white, the p53-binding site in green, and the p53 peptide in red. The lateral chains of Phe 19, Trp 23, and Leu 26 of p53 are shown. b) p53 Leu 22 is replaced by a tyrosine residue, and the lateral chain is manually located in the structure of the p53–hdm2 complex. The backbone of the p53 peptide is shown in gray, and Lys 94 of hdm2 is represented. c) The different hdm2 residues (Leu 57, Phe 86, Ile 99, and Ile 103) surrounding Trp 23 of p53 are indicated, and their van der Waals surface is represented in green.

Characterization of the interface

A yeast two-hybrid screen[35] and immunoprecipitation experiments[36] were initially used to map the two contact regions between both proteins. The hdm2-binding domain on p53 was localized between residues 1 to 52,[35,36] and the p53-binding domain on hdm2 between residues 1 to 118.[35,36] Further studies using site-directed mutagenesis identified Leu 14, Phe 19, Leu 22, and Trp 23 as key p53 contact residues,[37] and a minimal hdm2-binding site on the p53 protein was mapped between residues 18 to 23.[38] The strength of the interaction (K_d) between p53 peptides and hdm2 fragments has been determined by several methods and K_d values between 60 and 700 nM have been obtained depending on the length of these fragments and the methodology used.

The availability of the structure of a p53 peptide (residues 15–29) in complex with an hdm2 fragment (residues 17–125) permits a more detailed analysis of the interface (Figure 4a).[39] The p53-binding site on the hdm2 protein is a cleft about 25 Å

 © 2006 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim

long and 10 Å wide. In the bound p53 peptide, residues 19 to 25 form an α helix, and residues 17, 18, and 26–29 take a more extended conformation. The structure of the bound p53 peptide is stabilized by several intramolecular hydrogen bonds. This first observation indicates that hdm2 is the only one of the two proteins to possess a well-defined pocket. Inhibitors then have to be designed in such a way that they mimic p53. The calculated accessible surface area buried at the interface of hdm2 and p53 is about 660 $Å^2$ and 809 $Å^2$, respectively. Therefore, the interface between these two proteins is not excessively large, and it can accommodate drugs of standard size (see above). The determination of the planarity[8] of the hdm2 contact region is 3.1. This confirms that the contact region is not flat, but twisted in agreement with the presence of the above-described pocket. NMR experiments show that p53-derived peptides do not form a well-defined structure in solution;[40,41] this suggests that the p53 fragments only adopt the observed helical conformation when bound to hdm2. This structural organization of p53 upon binding is associated with a decrease in entropy, and experimental data give a change in entropy of $-40.4\,cal\,mol^{-1}$ for the binding of a p53 fragment to hdm2.[42] Upon p53 binding, conformational changes are also detected within the hdm2 protein.[43,44] The interaction between p53 and hdm2 is essentially hydrophobic, and 70% of the atoms at the interface are nonpolar. The three amino acids Phe 19, Trp 23, and Leu 26 from p53 are located on the same side of the helix, and their lateral chain point towards the hdm2 protein (Figure 4a). These amino acids make several interactions with hydrophobic hdm2 residues (Leu 54, Leu 57, Ile 61, Met 62, Tyr 67, Val 75, Val 93, Phe 86, Ile 99, Phe 91, and Ile 103). Only three direct hydrogen bonds are present at the interface: (p53 Phe 19)···(hdm2 Gln 72), (p53 Trp 23)···(hdm2 Leu 54), and (p53 Asn 29)···(hdm2 Tyr 100), and there is no water molecule bridging the two contact regions. This suggests high packing at the interface. Indeed, the gap volume[45] between both proteins is 892 $Å^3$, and the gap-volume index (the ratio between the gap volume and the interface-accessible surface area)[8] is 0.61 Å.

Altogether, the structural study of the p53–hdm2 interface suggests that it is likely to be a druggable target. It fits most of the criteria of the decision tree presented in Figure 1 (except for its high shape complementarity). Furthermore, as the p53 contact region is formed by only one segment of contiguous amino acids, peptides that mimic p53 can be used to establish or confirm a pharmacophore model and to study the effect of the inhibition of the p53–hdm2 interaction in tumor cells.

Establishment of a pharmacophore model and its validation

The structure of p53 in complex with hdm2[39] and initial data obtained with p53-derived peptides[35,38] indicate that peptides can be used to study this interaction to establish a pharmacophore model. Phage-display experiments[46] allowed the identification of a 12-mer phage-derived peptide **2**, which is 29-fold more potent than the wild-type peptide **1** (Table 2).[47]

Table 2. Example of peptidic inhibitors used as tool compounds for studying the p53–hdm2 interaction.

Peptide	Sequence[a]	IC_{50} [μM][b]
1	COOH-Gln-Glu-Thr-Phe^{19}-Ser-Asp-Leu-Trp^{23}-Lys-Leu-Leu^{26}-Pro-NH_2	8.7
2	COOH-Met-Pro-Arg-Phe^{19}-Met-Asp-Tyr-Trp^{23}-Glu-Gly-Leu^{26}-Asn-NH_2	0.3
3	COOH-Phe^{19}-Met-Asp-Tyr-Trp^{23}-Glu-Gly-Leu^{26}-NH_2	8.9
4	COOH-Phe^{19}-Met-Aib-Tyr-Trp^{23}-Glu-Ac_3c-Leu^{26}-NH_2	2.2
5	COOH-Phe^{19}-Met-Aib-Pmp-$6ClTrp^{23}$-Glu-Ac_3c-Leu^{26}-NH_2	0.005

[a] The position of the three key residues Phe 19, Trp 23, and Leu 26 is indicated. Aib = α-amino isobutyric acid, Ac_3c = 1-amino-cyclopropanecarboxylic acid, Pmp = phosphonomethylphenylalanine, 6ClTrp = 6-chlorotryptophan. [b] Determined by a competition assay.[48]

Peptide **2** was truncated to eight residues to give peptide **3**, which has activity in the micromolar range.[47] Notably, further deletions of peptide **3** which remove the essential residues Phe 19 or Leu 26 induce a dramatic drop in activity. As short peptides are usually very flexible in solution, and as the bound p53 takes a well-ordered structure when bound to hdm2, the next step was to decrease the flexibility of peptide **3** to decrease the entropic penalty "paid" upon binding. The two nonnatural amino acids, α-amino isobutyric acid (Aib) and 1-amino-cyclopropanecarboxylic acid (Ac_3c), were used to fix the conformation of the peptides in solution.[48,49] Different peptides were synthesized, and the more potent peptide **4** was obtained (Table 2). NMR spectroscopic measurements confirm a higher pre-organization in solution for peptide **4**. This peptide was modified to determine whether its potency could be improved by making new interactions with the hdm2 protein. Tyr 22 was replaced by phosphonomethylphenylalanine (Pmp), and Trp 23 was replaced by 6-chlorotryptophan (6-Cl-Trp).[48] The modification at p53 Tyr 22 creates a salt bridge with the amino group of hdm2 Lys 94 (Figure 4b). The addition of a chlorine atom at position 6 on Trp 23 was used to fill a small hydrophobic cavity formed by the hdm2 residues Leu 57, Phe 86, Ile 99, and Ile 103 which is unoccupied in the p53–hdm2 complex (Figure 4c). The formation of these new contacts by Pmp 22 and 6-Cl-Trp 23 results in an approximate 440-fold increase in the potency of peptide **4** (compare data for peptides **4** and **5**, Table 2). This gain in potency is probably associated with more a favorable enthalpic contribution in the binding energy.

Altogether this study with the peptides shows that the key contacts made by p53 Phe 19, Trp 23, and/or Leu 26 are important for the binding of p53 to hdm2 and that nonpeptidic inhibitors should therefore mimic these important interactions. The work carried out with the peptides containing nonnatural amino acids also indicates that despite the high complemen-

 © 2006 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim

tarity of the interface, it is possible to create additional interactions with hdm2 (Pmp 22 and 6-Cl-Trp 23), thus enhancing the potency of the inhibitors. This could also be exploited with nonpeptidic inhibitors.

The peptides were also used to demonstrate that the inhibition of the p53–hdm2 interaction in tumor cells leads to activation of the p53 pathway. Three different strategies have been used to introduce the p53 peptides into cells. Peptide **2** was inserted into the *Escherichia coli* thioredoxin protein[50] or fused to the glutathione S-transferase protein,[51] and peptide **5** has been directly used without further modification.[52,53] The data obtained with these different tools reveal that inhibitors of the p53–hdm2 interaction stimulate p53 activity (as measured by the induction of p53-regulated genes) in different tumor cells. These results are expected, as prevention of the hdm2-mediated degradation of p53 should induce its accumulation in cells and as a consequence, its activation. The activation of p53 by the peptides induces either a cell cycle or apoptosis, depending on the tumor cell line. This reveals that p53–hdm2 interaction inhibitors have an anti-proliferative effect and therefore behave as anticancer drugs.

The peptides were also used to study the effect of inhibiting the p53–hdm2 interaction in vivo. A p53 peptide (residues 16–27) was linked to the Tat transduction sequence and was used in New Zealand White Rabbits with intraocular retinoblastoma.[54] Injections of this peptide into the interior chamber induced tumor regression, and apoptosis was observed. This effect is specific to the tumor cell, as the peptide induced damage only to the tumor and not to the surrounding ocular tissues (lens, cornea, retina, etc.). These in vivo experiments suggest that inhibitors of the p53–hdm2 interaction have anticancer activity in vivo and, in addition, may not be toxic to nontumor tissues. This latter information is important, as inhibitors of the p53–hdm2 interaction also activate p53 in nontumor cells.[53]

Biological validation is a key step in any drug-discovery program because, even if a protein–protein interaction is a "top" drug target for medicinal chemistry, its inhibition should lead to the expected biological output. In the case of the p53–hdm2 interaction, the results obtained both in vitro and in vivo tend to demonstrate that inhibitors of this interaction will exert an anticancer activity in at least some tumors.

The synthesis of low-molecular-weight compounds

For many years, the only synthetic low-molecular-weight inhibitors of the p53–hdm2 interaction published were compounds that are not very potent. Only chalcone derivatives **6**,[44] some polycyclic compounds **7**,[55] and sulfonamides **8**[56] were described (Figure 5). A fungal metabolite, chlorofusin (**9**) was also described as an inhibitor of the p53–hdm2 interaction.[57] Finally, 1,4-benzodiazepine-2-ones such as **10** were proposed from a computational approach.[58]

These data were not very encouraging, and despite the attractiveness of this approach, it seemed not only that the druggability of the p53–hdm2 interaction was not as good as predicted by the structural analysis of the interface, but that obtaining potent low-molecular-weight inhibitors was not an attainable goal. However, scientists at Hoffmann–La Roche recently demonstrated the feasibility of inhibiting the p53–hdm2 interaction with low-molecular-weight compounds. Since the publication of the first reports on peptidic inhibitors of the p53–hdm2 interaction, it took about 10 years to obtain such results! By screening a diverse library of synthetic chemicals, Vassilev et al. were able to identify *cis*-imidazolines such as **11** (Figure 5), which they optimized for potency and specificity.[59] These compounds bind at the p53-binding site on hdm2, and their different substitutions mimic the key contacts made by p53 Phe 19, Trp 23, and Leu 26 (Figure 6). Furthermore, the halogen (Cl or Br) present on one of their phenyl groups mimics the chlorine atom of 6-Cl-Trp in peptide **5**. Finally, these mole-

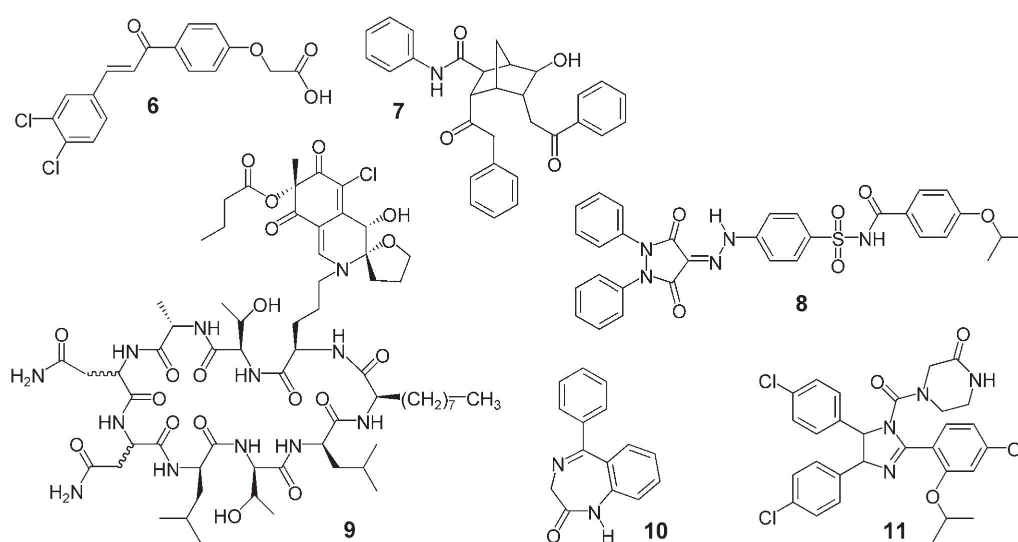


Figure 5. Low-molecular-weight inhibitors of the p53–hdm2 interaction: chalcone derivative **6**,[44] polycyclic compound **7**,[55] sulfonamide **8**,[56] chlorofusin (**9**), 1,4-benzodiazepine-2-one **10**,[58] and *cis*-imidazoline **11**.[59]

 © 2006 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim

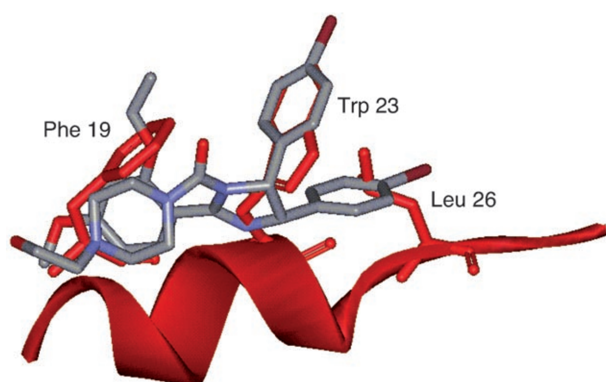


Figure 6. Binding mode of *cis*-imidazoline and p53 peptide. The structures of the *cis*-imidazoline–hdm2 complex[59] and the p53–hdm2 complex[39] are superimposed. Only the bound *cis*-imidazoline and the p53 peptide (in red) are represented. The lateral chains of Phe 19, Trp 23, and Leu 26 of p53 are shown.

cules are built up around a heterocycle and have a rigid conformation that minimizes the entropic contribution upon binding. Their potency (IC_{50}), measured in a competition assay, is in the 100–300 nM range. These compounds are active in various tumor cells (IC_{50} between 1 and 2 μM), where they induce activation of the p53 pathway. More importantly, they show efficacy as single agents in a tumor model in mice. In particular, compound **11**, (Figure 5) given orally at a dose of 200 mg kg^{-1} twice daily for 20 days, inhibits 90% of tumor growth (that is, of cells overexpressing hdm2). This treatment does not induce toxicity as measured by bodyweight measurements and necropsy. These data are highly encouraging, and it will be very exciting to see the effect of these molecules—or of their follow-ups—in the clinic.

7. Summary and Outlook

The design of inhibitors of protein–protein interactions is a hot topic in drug discovery today because many protein interfaces are exciting targets for pharmaceutical companies. However, one should be cautious with any assumption that the design of protein–protein interaction inhibitors will be a new Eldorado for the pharmaceutical industry, or conversely, that drug-discovery programs based on protein–protein interactions should be avoided because of the low probability of obtaining potent inhibitors. Protein interfaces are quite unique, and the only way to decide whether an interface is a "good" or "bad" target for drug discovery is to carry out a careful analysis of its structure before starting any drug-discovery activity. This should help in the selection of better targets, thereby decreasing the risk of time and resources invested in projects that do not deliver the expected molecules. The p53–hdm2 interaction is one example of the interfaces which have been successfully targeted with low-molecular-weight compounds (see also Table 1). Many other protein–protein interactions are under investigation, and it is likely that new inhibitors of protein–protein interaction will be described in the future.

Keywords: drug design · hdm2 · p53 · protein interfaces · protein–protein interactions

[1] A. L. Hopkins, C. R. Groom, *Nat. Rev. Drug Discovery* **2002**, *1*, 727–730.
[2] A. Fersht, *Enzyme structure and mechanism*, Freeman, New York, **1985**.
[3] P. Chène, *Expert Opin. Ther. Targets* **2003**, *7*, 453–461.
[4] S. Li, C. M. Armstrong, N. Bertin, H. Ge, S. Milstein, M. Boxem, P. O. Vidalain, J. D. Han, A. Chesnau, T. Hao, D. S. Goldberg, *Science* **2004**, *303*, 540–543.
[5] B. Kleizen, I. Braakman, *Curr. Opin. Cell Biol.* **2004**, *16*, 343–349.
[6] I. M. A. Nooren, J. M. Thornton, *EMBO J.* **2003**, *22*, 3486–3492.
[7] R. P. Bahadur, P. Chakrabarti, F. Rodier, J. Janin, *J. Protein Chem.* **2003**, *53*, 708–719.
[8] S. Jones, J. M. Thornton, *Proc. Natl. Acad. Sci. USA* **1996**, *93*, 13–20.
[9] L. Lo Conte, C. Chothia, J. Janin, *J. Mol. Biol.* **1999**, *285*, 2177–2198.
[10] S. J. Wodak, J. Janin, *Adv. Protein Chem.* **2003**, *61*, 9–73.
[11] I. M. Nooren, J. M. Thorton, *J. Mol. Biol.* **2003**, *325*, 991–1018.
[12] N. Brooijmans, K. A. Sharp, I. D. Kuntz, *J. Protein Chem.* **2002**, *48*, 645–653.
[13] W. L. DeLano, *Curr. Opin. Struct. Biol.* **2002**, *12*, 14–20.
[14] P. Chakrabarti, J. Janin, *J. Protein Chem.* **2002**, *47*, 334–342.
[15] J. Janin, *Structure* **1999**, *7*, R277–279.
[16] C. J. Tsai, S. L. Lin, H. J. Wolfson, R. Nussinov, *Crit. Rev. Biochem. Mol. Biol.* **1996**, *31*, 127–152.
[17] T. A. Larsen, A. J. Olson, D. S. Goodsell, *Structure* **1998**, *6*, 421–427.
[18] Y. Ofran, B. Rost, *J. Mol. Biol.* **2003**, *325*, 377–387.
[19] C. Cole, J. Warwicker, *Protein Sci.* **2002**, *11*, 2860–2870.
[20] J. A. Wells, *Proc. Natl. Acad. Sci. USA* **1996**, *93*, 7–12.
[21] T. R. Gadek, J. B. Nicholas, *Biochem. Pharmacol.* **2003**, *65*, 1–8.
[22] J. J. Schwartz, S. Zhang, *Curr. Opin. Mol. Ther.* **2000**, *2*, 162–167.
[23] A. Velazquez-Campoy, I. Luque, E. Freire, *Thermochim. Acta* **2001**, *380*, 217–227.
[24] K. H. Vousden, X. Lu, *Nat. Rev. Cancer* **2002**, *2*, 594–604.
[25] T. Soussi, K. Dehouche, C. Beroud, *Hum. Mutat.* **2000**, *15*, 105–213.
[26] S. M. Picksley, D. P. Lane, *Bioessays* **1993**, *15*, 689–690.
[27] X. Wu, J. H. Bayle, D. Olson, A. J. Levine, *Genes Dev.* **1993**, *7*, 1126–1132.
[28] J. Momand, G. P. Zambetti, D. C. Olson, D. George, A. J. Levine, *Cell* **1992**, *69*, 1237–1245.
[29] R. Honda, H. Yasuda, *Oncogene* **2000**, *19*, 1473–1476.
[30] S. Fang, J. P. Jensen, R. L. Ludwig, K. H. Vousden, A. M. Weissman, *J. Biol. Chem.* **2000**, *275*, 8945–8951.
[31] J. Roth, M. Dobbelstein, D. A. Freedman, T. Shenk, A. J. Levine, *EMBO J.* **1998**, *17*, 554–564.
[32] J. Momand, D. Jung, S. Wilczynski, J. Niland, *Nucleic Acids Res.* **1998**, *26*, 3453–3459.
[33] B. Eymin, S. Gazzeri, C. Brambilla, E. Brambilla, *Oncogene* **2002**, *21*, 2750–2761.
[34] D. Polsky, B. C. Bastian, C. Hazan, K. Melzer, J. Pack, A. Houghton, K. Busam, C. Cordon-Cardo, I. Osam, *Cancer Res.* **2001**, *61*, 7642–7646.
[35] J. D. Oliner, J. A. Pietenpol, S. Thiagalingam, J. Gyuris, K. W. Kinzler, B. Vogelstein, *Nature* **1993**, *362*, 857–860.
[36] J. Chen, V. Marechal, A. J. Levine, *Mol. Cell. Biol.* **1993**, *13*, 4107–4114.
[37] J. Lin, J. Chen, B. Elenbaas, A. J. Levine, *Genes Dev.* **1994**, *8*, 1235–1246.
[38] S. M. Picksley, B. Vojtesek, A. Sparks, D. P. Lane, *Oncogene* **1994**, *9*, 2523–2529.
[39] P. H. Kussie, S. Gorina, V. Marechal, B. Elenbaas, J. Moreau, A. J. Levine, N. P. Pavletich, *Science* **1996**, *274*, 948–953.
[40] M. J. J. Blommers, G. Fendrich, C. Garcia-Echeverria, P. Chène, *J. Am. Chem. Soc.* **1997**, *119*, 3425–3426.
[41] M. Uesugi, G. L. Verdine, *Proc. Natl. Acad. Sci. USA* **1999**, *96*, 14801–14806.
[42] Z. Lai, K. R. Auger, C. M. Manubay, R. A. Copeland, *Arch. Biochem. Biophys.* **2000**, *381*, 278–284.
[43] O. Schon, A. Friedler, M. Bycroft, S. M. V. Freund, A. R. Fersht, *J. Mol. Biol.* **2002**, *323*, 491–501.
[44] R. Stoll, C. Renner, S. Hansen, S. Palme, C. Klein, A. Belling, W. Zeslawski, M. Kamionka, T. Rehm, P. Muhlhahn, R. Schumacher, F. Hesse, B. Kaluza, W. Voelter, R. A. Engh, T. A. Holak, *Biochemistry* **2001**, *40*, 336–344.
[45] R. A. Laskowski, *J. Mol. Graphics* **1995**, *13*, 323–330.

 © 2006 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim

[46] V. Bottger, A. Bottger, S. F. Howard, S. M. Picksley, P. Chène, C. Garcia-Echeverria, H. K. Hochkeppel, D. P. Lane, *Oncogene* **1996**, *13*, 2141–2147.
[47] A. Bottger, V. Bottger, C. Garcia-Echeverria, P. Chène, H. K. Hochkeppel, W. Sampson, K. Ang, S. F. Howard, S. M. Picksley, D. P. Lane, *J. Mol. Biol.* **1997**, *269*, 744–756.
[48] C. Garcia-Echeverria, P. Chène, M. J. Blommers, P. Furet, *J. Med. Chem.* **2000**, *43*, 3205–3208.
[49] R. Banerjee, G. Basu, P. Chène, S. Roy, *J. Pept. Res.* **2002**, *60*, 88–94.
[50] A. Bottger, V. Bottger, A. Sparks, W. L. Liu, S. F. Howard, D. P. Lane, *Curr. Biol.* **1997**, *7*, 860–869.
[51] C. Wasylyk, R. Salvi, M. Argentini, C. Dureuil, I. Delumeau, J. Abecassis, L. Debussche, B. Wasylyk, *Oncogene* **1999**, *18*, 1921–1934.
[52] P. Chène, J. Fuchs, J. Bohn, C. Garcia-Echeverria, P. Furet, D. Fabbro, *J. Mol. Biol.* **2000**, *299*, 245–253.
[53] P. Chène, J. Fuchs, I. Carena, P. Furet, C. Garcia Echeverria, *FEBS Lett.* **2002**, *529*, 293–297.
[54] J. W. Harbour, L. Worley, D. Ma, M. Cohen, *Arch. Ophthalmol.* **2002**, *120*, 1341–1346.
[55] J. Zhao, M. Wang, J. Chen, A. Luo, X. Wang, M. Wu, D. Yin, Z. Liu, *Cancer Lett.* **2002**, *183*, 69–77.
[56] P. S. Galatin, D. J. Abraham, *J. Med. Chem.* **2004**, *47*, 4163–4165.
[57] S. J. Duncan, S. Gruschow, D. H. Williams, C. McNicholas, R. Purewal, M. Hajek, M. Gerlitz, S. Martin, S. K. Wrigley, M. Moore, *J. Am. Chem. Soc.* **2001**, *123*, 554–560.
[58] N. Majeux, M. Scarsi, A. Caflisch, *J. Protein Chem.* **2001**, *42*, 256–268.
[59] L. T. Vassilev, B. T. Vu, B. Graves, D. Carvajal, F. Podlaski, Z. Filipovic, N. Kong, U. Kammlott, C. Lukacs, C. Klein, N. Fotouhi, E. A. Liu, *Science* **2004**, *303*, 844–848.
[60] Y. C. Cheng, W. H. Prusoff, *Biochem. Pharmacol.* **1973**, *22*, 3099–3108.

Received: January 4, 2006
Published online on February 17, 2006

 © 2006 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim