

Chapter 2

Protein–Protein Interaction Inhibitors: Case Studies on Small Molecules and Natural Compounds

Stefania Ferrari, Federica Pellati and Maria Paola Costi

Abbreviations

A β	β -amyloid
A β PP	β -amyloid protein precursor
Aib	α -aminoisobutyric acid
BIR	Baculoviral inhibitory repeat
c-kit	Tyrosine kinase receptor
dTMP	2'-deoxythymidine-5'-monophosphate
dUMP	2'-deoxyuridine-5'-monophosphate
FRET	Förster resonance energy transfer
GAPs	GTPase-activating proteins
GEFs	Guanine nucleotide exchange factors
HDM2	Human double minute 2
HeLa	Human cervical carcinoma cancer cell line
HTS	High-throughput screening
hTS	Human thymidylate synthase
IAPs	Inhibitors of apoptosis proteins
MAP	Mitogen-activated protein
MDM2	Mouse double minute 2
NCI	National Cancer Institute
NSAIDs	Non-steroidal anti-inflammatory drugs
O-PHDEs	Oxy-polyhalogenated diphenyl ethers
PAC	Proteasome assembling chaperone
PBD	Polo-box domain
PDPA	1,3-propanediphosphonic acid

S. Ferrari · F. Pellati · M. P. Costi (✉)
Department of Life Sciences, University of Modena and Reggio Emilia,
Via Campi 183, 41125 Modena, Italy
e-mail: mariapaola.costi@unimore.it

S. Ferrari
e-mail: stefania.ferrari@unimore.it

F. Pellati
e-mail: federica.pellati@unimore.it

PI3K	Phosphoinositol-3'-kinase
PPIs	Protein-protein interactions
PS	Presenilin
SCF	Stem cell factor
Tcf4	T-cell factor 4
XIAP	X-linked Inhibitor of apoptosis protein

2.1 Introduction

Many biological functions involve the formation of protein-protein complexes, and the inhibition of this process has garnered significant interest in pharmaceutical research investigating novel therapies for several human diseases. From an evolutionary perspective, proteins have evolved to optimize and differentiate their functions, a process that is mediated by the modulation of the interacting surfaces. Protein-protein interactions (PPIs) represent another dimension of the structural properties of proteins and are the essence of the interactome, the ensemble of all complexes generated through PPIs present at the cellular level. The aggregation states of a protein monomer, for example, can cover a wide range of complexes; the same protein in monomeric form may have a different function with respect to its aggregated form [1]. This phenomenon enlarges the biological space considerably and increases its complexity. PPIs can occur between two (or more) identical protein sequences (homo-oligomers) or between two different protein sequences (hetero-oligomers) [2]. PPIs can also be distinguished on the basis of the stability of the complex: there are permanent complexes (structurally obligate oligomers generally fall into this category) and transient complexes. In structurally and/or functionally obligate oligomers, single monomers cannot exist as a long-lived entity or they are inactive *in vivo*. The multiple functions of proteins may account for their high structural complexity. In the case of enzymes, their catalytic functions are mostly located in the active site region, while the other protein domains, both solvent accessible or non-solvent accessible, may be responsible for different functions. Many pathological processes are governed by PPIs; therefore, designing molecules that interfere with the formation of these complexes is one of the recent challenges in drug design [2].

So far, only a minimal fraction of the estimated 650,000 PPIs that comprise the human interactome are known, and only a few categories have been described [3]. These interactions control processes involved in both normal and pathological pathways, which include signal transduction, cell adhesion, cellular proliferation, growth, differentiation, viral self-assembly, programmed cell death, and cytoskeleton structure. PPIs that play a role in signal transduction or cell regulation are often temporary and weak [4, 5].

PPIs are tentatively organized as follows: (a) PPIs (high molecular mass), which include membrane receptors; (b) oligomeric proteins (monomer-monomer

interactions) that can be homo- or heteromeric; (c) peptide–protein interactions; and (d) protein–antibody interactions (immunoconjugates or immunocomplexes). In category (a), we include those proteins that often use the complex networks of interactions to produce sophisticated signaling networks that are capable of well-tuned and highly adaptive responses to environmental stimuli, such as programmed cell death [6–9].

Because signal transduction is related to how the extracellular environment exerts its effect on cellular status and function, many steps in signal transduction pathways involve proteins that represent potential drug targets [10]. Category (b) is mostly related to the self-assembly of proteins in different aggregates depending on the local cellular environment [11]. Category (c) includes all of the regulatory networks that are part of the peptidome (composed of all the small peptides that have often unknown regulatory functions in the cells) [12]. Category (d) includes antibody–antigen complexes, which are a well-described type of protein–protein interaction. This complex is characterized by highly specific molecular recognition. At the moment, this type of protein–protein interaction has not been studied from a drug discovery perspective but is mostly important from a functional point of view.

The experimental detection of protein complexes is very common. Biologists characterize new protein complexes to identify the total number of ligands that establish interactions with the target protein by co-precipitating the ligand and protein in immunocomplexes, using affinity tagging and pull-down assays or using two hybrid methods to understand their functions [13]. However, the mechanistic role of the identified complexes is not easy to establish. To this aim, unnatural amino acids have been inserted through molecular biology approaches using orthogonal acyl-t-RNA transferases to generate mutated proteins with altered functions [14]. The possibility of studying the biological role of PPIs in a native cellular environment is fundamental to avoid artifacts and drive data interpretations. X-ray crystallographers generate protein crystals that often show oligomeric structures. At the moment, crystallization experiments can only be performed at high protein concentrations, and therefore, protein aggregation depends on the equilibrium constants that govern the relative affinity of the monomers. Other biophysical methods are also important in efforts to understand the multimeric protein state. Among them, NMR can be used to determine structural information about the aggregation state of a protein. Although these experimental approaches are not performed in conditions that are fully physiologically relevant, they can be used to establish that the protein can self-assemble with a recognition code that is not easy to rationalize. So far, only a small number of complexes have been considered as druggable targets. Such intricate biological systems cannot be cost-effectively tackled using conventional high-throughput screening (HTS) methods. Some researchers argue that accomplishing this goal will be difficult, if not impossible, because the target binding site is a multipoint interface between two proteins with sufficient stabilizing force to bind two molecules together. Therefore, there is competition between the ligand and the partner protein. However, the development of PPIs inhibitors is becoming more and more feasible [15].

The increased enthusiasm for PPI inhibitors is not just restricted to preclinical programs, and companies are advancing a handful of such compounds through clinical trials [16].

2.2 Characterizing the Binding Site

Proteins interact in complicated ways because their shapes are so vastly complex. Amino acid side chains that extend from the body of the molecule create cavities or bumps of different shapes and sizes. Proteins maximally exploit this structural diversity, producing binding pockets and recognition sites with varying degrees of specificity. In general, a core interaction area and a limit interaction area are recognized in PPIs. Core interaction area involves the highest conserved residues, while the limit interaction area is less crucial for the interaction and is less conserved.

Most current drugs target the binding site of a protein that naturally binds the substrates or are very close to those sites. In fact, most of the compounds that are tested have affinity for the active site. Enzyme interface inhibitors usually affect the catalytic function as a consequence of the fact that the aggregation state often directly influences catalytic function itself as in the case of HIV protease [17]. Most of the work performed has not been able to show experimental details at the structural level to characterize the binding site of protein–protein interaction inhibitors [18]. The characterization of such interfaces represents the true barrier to effectively understanding the structural nature of PPIs and is consequently a major obstacle for designing modulating agents that can bind to those interfaces. In general, interaction interfaces are considered to be flat and rigid; in fact, they are dynamic and can be more convoluted in solution than they appear in co-crystal structures. This complexity causes some difficulties in the selection of the suitable template conformation. The properties that describe the interactions include the size and shapes of the interface, the accessible surface area, hydrophobicity, and the propensity of residues to participate in hydrophobic or hydrogen bond interactions.

In Fig. 2.1, an example of an interface binding pocket that usually binds other proteins and forms heterodimeric interfaces is shown [19]. The Nef protein of HIV was studied via a combined virtual and experimental screening method, and no previously known inhibitors could be used as a starting point in a structure-based research program that targets an SH3-binding surface of the HIV type I Nef protein. High-throughput docking and the application of a pharmacophoric filter, on the one hand, and the search for analogy, on the other hand, identified drug-like compounds that were further confirmed to bind Nef in the micromolar range.

Computational chemists have developed different tools designed to specifically study PPI inhibitors, including machine-learning-based models, which are mainly decision trees that use a dataset of known PPI inhibitors and of regular drugs to determine a global physicochemical profile for putative PPI inhibitors [20]. The

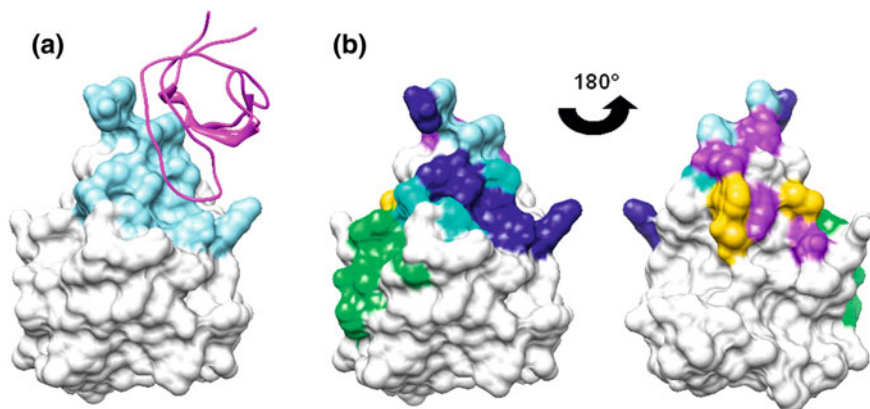


Fig. 2.1 HIV-1 Nef binding surfaces. **a** X-ray structure of the Nef-SH3Hck complex (Protein DataBank ID code: 1AVZ). The Nef surface is represented in light gray with the SH3 binding region in light blue; the SH3 domain is shown in magenta ribbon. **b** Residues of the Nef core domain molecularly defined to be involved either directly or indirectly in cellular protein interactions and Nef functions are colored as follow: (i) SH3 binding region in blue (“proline-rich region” in sky blue, “RT loop binding region” in cyan, all the remaining residues in navy blue); (ii) p21 associated kinase 1/2 binding residues in purple; (iii) CD4 binding residues in green; (iv) Dyn2 and human thioesterase binding residues in yellow.

statistical analysis of the properties of effective PPI inhibitors included in the dataset, in relation to the compound’s propensity to bind at the interface, unravels two important molecular descriptors for PPI inhibitors: specific molecular shapes and the presence of a privileged number of aromatic interactions that represent dominating aspects. These aspects are analyzed in another chapter within this book.

Attempts have been made to characterize the interacting interfaces in PPIs involved in inhibitors binding. A large variety of these PPI databases [21–25] depict PPIs at a structural level (for a summary of these available databases refer to [26]). A new system has been proposed by Morelli and collaborators [27] in which the specific PPI chemical space is represented through the presentation of the protein–protein interaction inhibitor (2P2I) database (DB), a hand-curated database dedicated to the structures of PPIs with known inhibitors (Fig. 2.2). PPIs and protein/inhibitor interfaces have been analyzed in terms of geometrical parameters, atom and residue properties, the buried accessible surface area, and other biophysical parameters. The interfaces found in 2P2I DB were then compared to those of representative datasets of heterodimeric complexes. In particular, they were compared with the interfaces found in representative datasets of heterodimeric complexes from Bahadur and Zacharias [28] or from the ProtorP parameters (<http://www.bioinformatics.sussex.ac.uk/protorp/>). A new classification of PPIs with known inhibitors into two classes is proposed, depending on the number of segments present at the interface and corresponding to either a single secondary structure element or a more globular interaction domain. 2P2I DB complexes share

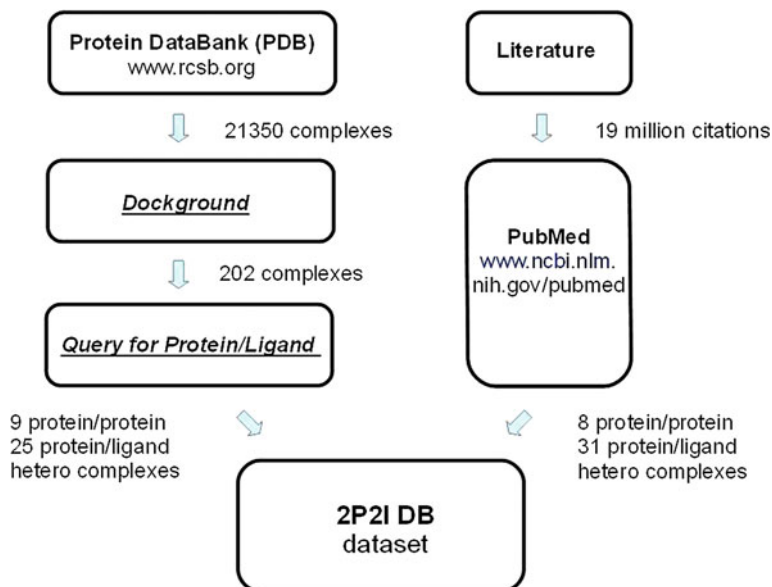


Fig. 2.2 2P2I database (DB), a hand-curated database dedicated to the structure of PPIs with known inhibitors (from [27])

global shape properties with standard transient heterodimer complexes, but their accessible surface areas are significantly smaller. No major conformational changes have been observed between the different states of the proteins. The interfaces are more hydrophobic than general PPI interfaces, with less charged residues and more non-polar atoms. Finally, 50 % of the complexes in the 2P2I DB dataset possess more hydrogen bonds than typical protein–protein complexes. Potential areas of study for the future have been proposed and include a new classification system consisting of specific families and the identification of PPI targets with high druggability potential based on key descriptors of the interaction.

However, proteins may have other roles, such as regulatory functions that modulate the expression of other proteins performed through the interaction of the protein with an mRNA molecule. This new generation of drugs can act as competitive antagonists or make much more subtle alterations through allosteric inhibitions, by only disrupting the way in which a protein interacts with other specific proteins (Fig. 2.1). These considerations suggest that the allosteric inhibitors or PPI interactions may generate new mechanisms of inhibition of the same protein with respect to active site inhibitors. In general, PPI inhibitor must have high affinity for monomer surface A and must repel monomer surface B where AB is the target dimeric protein. This concept is still not widely considered in the drug design approach of new PPIs inhibitors.

2.3 Structural Features of Protein–Protein Interaction Inhibitors

The emergence of PPI development programs has been driven by technological and conceptual advances in understanding PPI druggability and the types of molecules that can be used to block interactions. Biologics, in many ways, are more natural candidates for the inhibition of PPIs because they more likely resemble a natural PPI partner-like interface peptides mimic and their large size offers more opportunities to block an interaction and hit various hotspots (critical spots on the protein surface that are relevant for the interaction with a physiological partner protein). A large variety of compounds with nearly unrelated structures have been shown to interfere with the same protein–protein interface. These compounds may bind to the enzyme active site, if the active site is naturally located at the interface of the two or more monomers, they may bind to allosteric binding sites with respect to the substrate-binding sites. Their structures are not conserved, and there is no common general structure–activity relationship (SAR) that can recognize these compounds. However, systematic analysis has been performed to classify the protein–protein interaction inhibitors (2P2Is). Morelli et al. [26, 27] reported that the 2P2I DB (<http://2p2idb.cnrs-mrs.fr>) contained a total of 17 protein/protein complexes corresponding to 14 families and 56 small-molecule inhibitors bound to the corresponding target (Fig. 2.3).

In the remaining part of this chapter, we will describe success stories within the PPI studies and natural compounds known as PPIs.

2.4 Successful and Recent Stories: γ -Secretase, Caspase 9, XIAP/BIR3, SMAC System, p21-Ras Oncoprotein, Human Thymidylate Synthase

Several recent success stories indicate that protein–protein interfaces might be more tractable than previously thought. These studies discovered small molecules that bind with drug-like potencies to “hotspots” on the contact surfaces involved in PPIs. Remarkably, these small molecules bind deeper within the contact surface of the target protein and bind with much higher efficiencies compared with the contact atoms of the natural protein partner. Some of these small molecules are now making their way through clinical trials.

2.5 γ -Secretase

γ -Secretase is a high molecular weight (HMW) membrane protein complex that includes presenilin (PS), nicastrin, Aph-1, and Pen-2. γ -Secretase is an

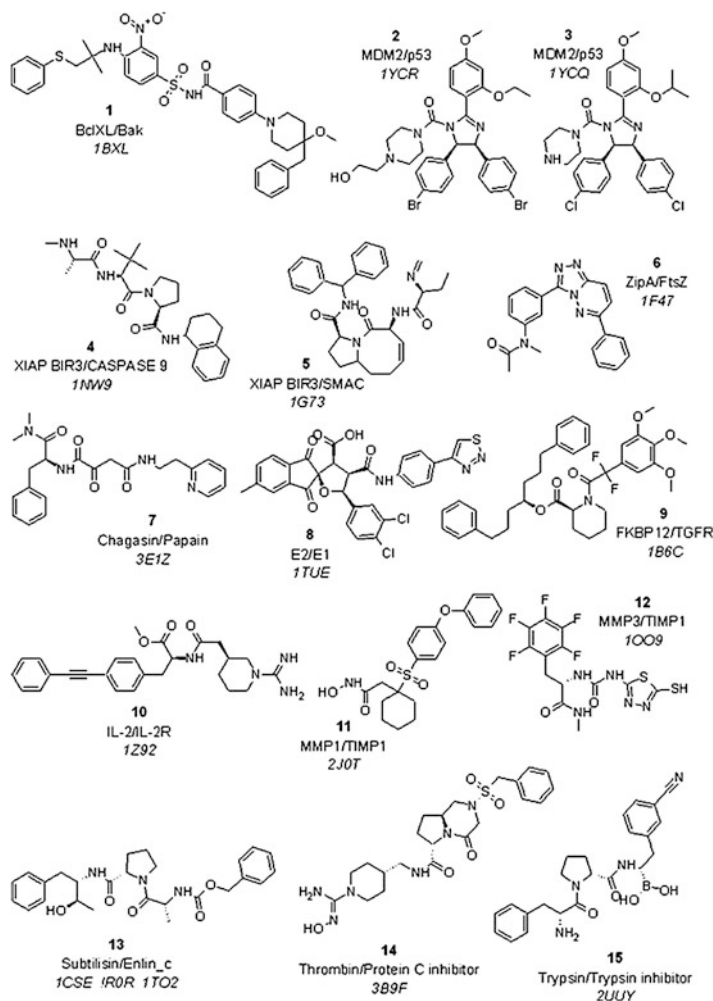


Fig. 2.3 List of representative small-molecule inhibitors for each protein–protein complex in the 2P2I DB. Only the inhibitor used to define the subset of the interface at 4.5 Å around the ligand is shown. For each inhibitor, the name of the protein family, the PDB code of the complex, and the molecular weight of the ligand are indicated

intramembrane-cleaving aspartyl protease [29]. The catalytic activity of the γ -secretase complex centers on the presenilin-1 (PS-1) protein, which is perhaps most notable for its role in mediating the cleavage of the β -amyloid (A β) peptide from the amyloid protein precursor (A β PP) [30]. A β PP is cleaved initially by α - or β -secretase to generate membrane-bound C-terminal fragments (C83 and C99, respectively). α -Secretase activity appears to be mediated by the disintegrin and metalloprotease (ADAM) family members TACE and ADAM-10. β -secretase (BACE, Asp-2) was recently cloned and characterized as a novel membrane-bound

aspartyl protease. The C83 and C99 cleavage products serve as substrates for the γ -secretase, and A β (40) and A β (42) are generated from C99. This cleavage is rather unusual in that it occurs at a site within the putative transmembrane domain of A β PP. A β 42 is the major constituent of the amyloid plaques in the brain parenchyma of Alzheimer's disease patients (Fig. 2.4) [31].

The first class of γ -secretase inhibitors consisted of transition-state analogues designed to interact specifically with the diaspartyl active site of γ -secretase. Some of these inhibitors are peptidomimetics, which contain a non-hydrolyzable difluoro-alcohol moiety that resembles the transition state of the proteolysis carried out by aspartyl proteases or contain a non-hydrolyzable difluoro-ketone moiety that readily forms a hydrate resembling the aspartyl-protease-catalyzed transition state (Fig. 2.5). Moreover, it has been found that installing large, hydrophobic P1 substituents (such as *sec*-butyl or cyclohexylmethyl) into these transition-state mimics enhances their potency, indicating that there may be a relatively large complimentary S1 pocket in γ -secretase capable of accommodating these bulky substituents [32]. L-685,458 is a specific inhibitor of A β PP γ -secretase that contains a hydroxyethylene dipeptide isostere (Fig. 2.5) [31].

An α -helical peptide-based inhibitor has been described to bind PS fragments in a manner distinct from that of transition-state analogue inhibitors [29]. New inhibitor prototypes that would assume a helical conformation, such as short peptides based on the A β PP transmembrane domain, were designed. Evidence suggests that the A β PP transmembrane domain is helical upon its initial interaction with the γ -secretase complex. To achieve this conformational constraint, a well-known helix-inducing residue, α -aminoisobutyric acid (Aib), was incorporated. In this design, A β PP residues were judiciously swapped with Aib so that, upon helix formation, the Aib residues would be on one face of the helix and the APP residues would be on the other. Further, N-terminal *tert*-butoxycarbonyl, C-terminal methyl ester, and threonine *O*-benzyl protecting groups were retained to enhance cell permeability. These peptides blocked A β production from A β PP-transfected Chinese hamster ovary (CHO) cells in the low μ M range, with longer peptides showing higher potencies. Inhibition occurred at the γ -secretase level, as A β PP γ -secretase substrates were increased in a concentration-dependent manner consistent with the effects on A β production. Surprisingly, the enantiomers of

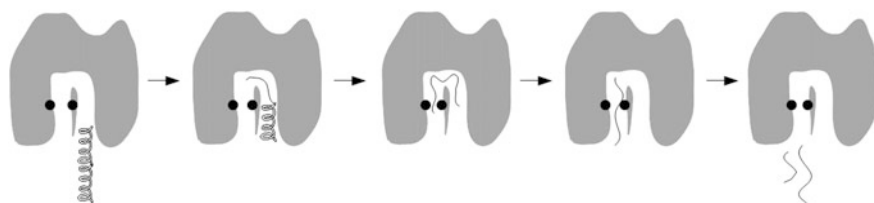


Fig. 2.4 Scheme of the enzymatic function of γ -secretase. The peptidic substrate carrying an α -helical transmembrane domain structure enters the “initial binding site”; then, it goes on to the catalytic site and undergoes an unwinding process. The substrates are then proteolyzed and the resulting fragments are liberated

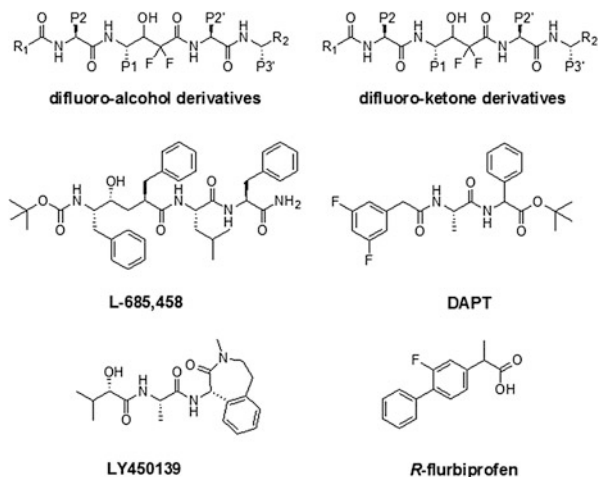
these compounds, with all D-amino acids, were equally or more potent than their L-peptide counterparts because both enantiomers are helical in solution [33].

Finally, a number of structurally diverse and potent γ -secretase inhibitors have been reported in addition to the classical transition-state analogues. One of these non-transition-state analogue γ -secretase inhibitors is DAPT {N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester} (Fig. 2.5) [29]. This inhibitor was discovered as a result of a sandwich ELISA screen of approximately 25000 compounds for their ability to inhibit cellular A β production. A chemical optimization program was initiated around one active compound, N-arylanaline ester, which emerged from this screen [34]. Other compounds that were identified included arylsulfonamides and benzodiazepines [35].

It is important to mention that this HMW membrane protein complex seems to have different sites for catalytic function, substrate binding, and inhibitor binding. Recent studies based on inhibitor cross-competition kinetics indicated the presence on γ -secretase of two inhibitor binding sites, one for binding to transition-state isosteres and the other for non-transition-state small-molecule inhibitors [36]. Moreover, because γ -secretase cleaves amide bonds within the transmembrane regions of its substrates via a poorly understood process of hydrolysis within a hydrophobic environment and because of its requirement for water, the active site of γ -secretase is thought to be in the protein interior to avoid the hydrophobic environment of the lipid bilayer. As a result, the integral membrane substrates should initially interact on the surface of the protease before entering the internal active site. Substrate-binding sites that are distinct from the active site are generally called exosites or substrate-docking sites. It is believed that helical peptide inhibitors bind to γ -secretase at the substrate-docking site [37].

One compound, LY450139 or Semagacestat, a benzolactam γ -secretase inhibitor (Fig. 2.5), has entered clinical trials. These studies have shown a dose-dependent decrease in the plasma but not the cerebral spinal fluid levels of A β .

Fig. 2.5 Structures of select γ -secretase inhibitors



However, Semagacestat had no cognitive or functional benefit for patients with mild to moderate Alzheimer's disease in a multi-center, double-blinded, randomized, placebo-controlled phase II study. In addition, it is disappointing that preliminary results from two phase III clinical trials showed that Semagacestat did not slow down disease progression and was associated with a worsening of clinical measures of cognition and the ability to perform the activities of daily life. Unfortunately, Semagacestat is also associated with an increased risk for skin cancer. Consequently, the clinical trial of LY450139 was halted [38, 39].

Although γ -secretase is in many ways an attractive target for Alzheimer's disease therapeutics, interference with Notch processing and signaling may lead to toxicities that preclude the clinical use of inhibitors of this protease. Nevertheless, hope remains that a γ -secretase inhibitor might lower $A\beta$ production in the brain enough to prevent $A\beta$ oligomerization and fibril formation while leaving enough Notch signaling intact to avoid toxic effects. This hope has stimulated the continuing clinical studies of LY450139, even though compounds in this general structural class have not demonstrated selective inhibition of $A\beta$ PP processing with respect to Notch [39–41].

In contrast, compounds that can modulate the enzyme to alter or block $A\beta$ production with little or no effect on Notch would bypass this potential roadblock to the development of therapeutics. Recent studies suggest that the protease complex contains allosteric binding sites that can alter substrate selectivity and the sites of substrate proteolysis [40].

Certain non-steroidal anti-inflammatory drugs (NSAIDs) can reduce the production of the highly aggregation-prone $A\beta$ 42 peptide and increase the level of a 38-residue form of $A\beta$. This pharmacological property is independent of the inhibition of cyclooxygenase. Enzyme kinetics studies and displacement experiments suggest that selective NSAIDs can be non-competitive with respect to the $A\beta$ PP substrate and to a transition-state analogue inhibitor, which also suggests an interaction with a site distinct from the active site and the docking site. While the biochemical mechanism of these NSAID γ -secretase modulators is unclear, the effect on $A\beta$ PP proteolysis resulting in lower $A\beta$ 42 and increased $A\beta$ 38 has been well documented. The site of cleavage within the Notch transmembrane domain is similarly affected, but this subtle change does not inhibit the release of the intracellular domain and thus does not affect Notch signaling. For this reason, these agents may be safer as Alzheimer therapeutics than inhibitors that block the active site or the docking site. Indeed, one compound, *R*-flurbiprofen (formerly flurizan, now called tarenflurbil) (Fig. 2.5), has recently advanced to phase III clinical trials in the USA. The enantiomer, *S*-flurbiprofen, is a COX inhibitor, whereas *R*-flurbiprofen modulates $A\beta$ production but is not a COX inhibitor. However, the potency of this drug candidate and other NSAIDs in terms of lowering $A\beta$ 42 raises questions about efficacy, and it will likely be important to better understand how these compounds interact with PS and develop second- and third-generation agents [40].

Another type of allosteric modulator includes compounds that resemble kinase inhibitors and interact with a nucleotide-binding site on the γ -secretase complex.

The discovery that ATP can increase $A\beta$ production in membrane preparations prompted the testing of a variety of compounds known to interact with ATP-binding sites on other proteins. In a focused screen, the kinase inhibitor Gleevec emerged as a selective cellular inhibitor of $A\beta$ production without affecting the proteolysis of Notch. In light of these findings, ATP and other nucleotides were tested for the effects on purified γ -secretase preparations and were found to selectively increase the proteolytic processing of a purified recombinant $A\beta$ PP-based substrate without affecting the proteolysis of the Notch counterpart. This and other evidence suggest that the γ -secretase complex contains a nucleotide-binding site and that this site allows the allosteric regulation of the γ -secretase-mediated processing of $A\beta$ PP with respect to Notch. Whether this regulation is physiologically important is unclear, but the pharmacological relevance is profound and may lead to the development of new therapeutic candidates for AD. This hope is tempered by the fact that γ -secretase cleaves numerous other type I membrane protein substrates. The use of agents that are selective for APP *versus* Notch may result in new long-term toxicities as a result of blocking the proteolysis of other substrates. To address this important issue, the development of more potent analogues that work by this mechanism will be critical [40].

2.6 Caspase 9, XIAP/BIR3, SMAC System

Apoptosis, or programmed cell death, originates from at least two different pathways: an intrinsic pathway initiated from mitochondrial membrane depolarization as a result of irreparable damage to the genome, microtubule disruption, and other factors, and an extrinsic pathway activated by tumor necrosis factor family death receptors. Both apoptotic pathways culminate in the activation of cysteine protease family members known as caspases. Caspase-9 is an initiator caspase in the intrinsic pathway. Upon the recruitment to a multiprotein structure called the apoptosome, procaspase-9 dimerizes into a catalytically active form that cleaves and activates procaspase-3 and procaspase-7 (Fig. 2.6) [42].

Apoptosis plays a crucial role in the homeostasis and development of living organisms. Deregulation of this mechanism is associated with many diseases, including several types of cancer.

In the apoptosis pathway, the inhibitors of apoptosis proteins (IAPs) are exploited by tumor cells to evade programmed cell death. The XIAP (X-linked inhibitor of apoptosis protein) is the most potent caspase inhibitor among the IAP protein family. XIAP contains three baculoviral inhibitory repeat (BIR) domains and a ring domain. This protein interacts with initiator caspase-9 and executioner caspase-3 and caspase-7 through its BIR3 and BIR2 domains, respectively. BIR3 inhibits caspase-9 by preventing dimerization, which is required for its catalytic activity. The search for new compounds that are able to disrupt the XIAP–caspase interaction has attracted the attention of scientific community as a promising strategy for cancer treatment (Fig. 2.6) [42, 43].

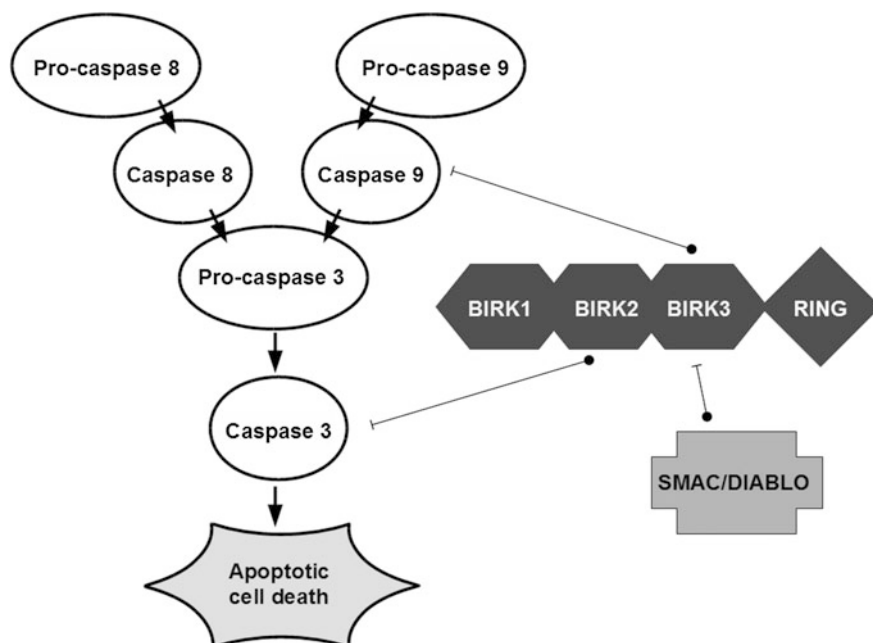


Fig. 2.6 Two different pathways to the programmed cell death, that is, apoptosis

Two broad approaches have been taken to develop clinical inhibitors of XIAP—antisense oligonucleotides and small-molecule inhibitors. Antisense oligonucleotides, aside from the disadvantages inherent to the method, are advantageous, as they target the entire protein, whereas small molecules bind a single domain. However, small-molecule inhibitors offer the potential of a more rapid inhibition of their target *in vivo* and a more predictable duration of action.

Antisense oligonucleotides directed against XIAP are being developed for the treatment for solid tumors and hematologic malignancies by Aegera pharmaceuticals (Montreal, Canada). The antisense molecule currently in clinical trials (AEG 35156) is a second-generation 19-mer antisense oligonucleotide that targets XIAP. It contains a mixed backbone of chemically modified DNA/RNA nucleotides. Antisense oligonucleotides inhibit their target by forming duplexes with the intracellular native mRNA. The duplexes recruit RNase H enzymes that cleave the native mRNA strand while leaving the antisense oligonucleotide intact. The antisense oligonucleotide is then released back into the cytosol where it is capable of inhibiting additional native mRNA molecules. In cultured cells, the uptake of antisense oligonucleotides requires transfection, infection, or electroporation protocols. In xenografts and patients, the intracellular uptake of antisense molecules is achieved after intravenous or subcutaneous administration, but the mechanism facilitating the uptake is not clear.

Although the xenograft data have been encouraging, the efficacy of antisense therapies in previous clinical trials has not matched the initial expectations. To

date, most antisense trials have indicated that these antisense oligonucleotides are safe, but their efficacy is modest. Given the redundancy in the IAP family, the selective targeting of XIAP may not produce an optimal effect. In this case, a different approach using pan-IAP inhibitors may be needed. The lack of a significant response to antisense oligonucleotides may also reflect a failure to achieve adequate target knockdown, as it is unknown how much knockdown is required to inhibit the function of the target. Finally, it remains unclear whether the effects of antisense therapies are related to the knockdown of their target or non-specific effects resulting from altered gene regulation. Given these concerns, XIAP antisense therapy may also be hampered in clinical trials. Therefore, there is great interest in developing small-molecule inhibitors of XIAP to overcome the limitations of antisense oligonucleotides [44].

The natural inhibitor of XIAP, SMAC/DIABLO (second mitochondrial activator of caspases/direct IAP-binding protein with Low PI), is a protein that is released from the mitochondria into the cytosol in response to apoptotic stimuli. SMAC removes the XIAP inhibition of caspase-9 by binding to the BIR3 domain of XIAP through the AVPI tetrapeptide present in the N-terminal part of SMAC. This interaction (AVPI/BIR3) has been determined unequivocally by X-ray crystallography [43].

Since the discovery of the SMAC protein in 2000, there has been an enormous interest in academic laboratories and pharmaceutical companies in the design of small-molecule SMAC mimetics [45]. Various design methods have been used to generate peptidomimetics and non-peptide SMAC mimetics with improved stability and enhanced drug-like properties. A number of research groups have reported the discovery of small-molecule BIR3 inhibitors by various methods, including peptidomimetic approaches, virtual screening/structure-based design, or the screening of natural products or synthetic libraries [46].

Using the AVPI structure (Fig. 2.7), a comprehensive study has been performed to determine which amino acids could be substituted without compromising the peptide-binding affinity. The alanine (first amino acid) and the proline (third amino acid) are the essential amino acid residues needed to preserve the activity of this tetrapeptide. However, the use of synthetic SMAC-derived peptides as therapeutic compounds is hindered by their limited cell permeability, proteolytic instability, and poor pharmacokinetics [43]. To address these limitations, a number of early studies employed a strategy to tether a carrier peptide to a SMAC-based peptide to facilitate intracellular delivery. It was shown that these relatively cell-permeable SMAC-based peptides can sensitize various tumor cells *in vitro* to the anti-tumor activity of Apo-2L/TRAIL, as well as chemotherapeutic agents such as paclitaxel, etoposide, SN-38, doxorubicin, and cisplatin [45].

Extensive chemical modifications of the AVPI peptide have been carried out in an effort to derive potent SMAC peptidomimetics. Furthermore, conformationally constrained, bicyclic SMAC mimetics were designed using a structure-based strategy (Fig. 2.7) [45].

It has been demonstrated that the natural SMAC protein forms a dimer and binds to XIAP protein constructs containing BIR2 and BIR3 domains with a much

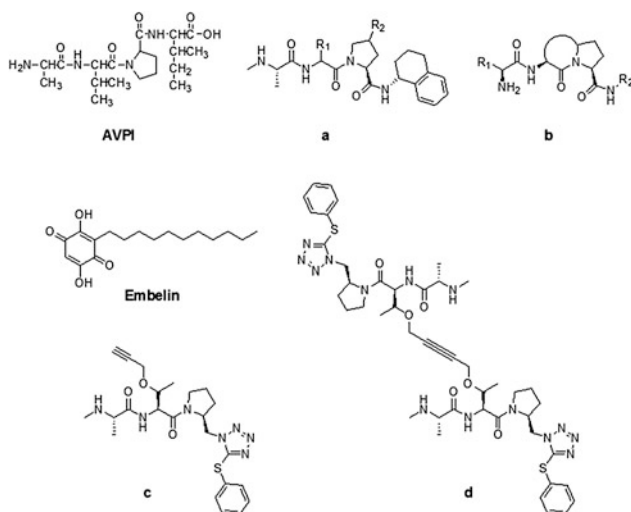


Fig. 2.7 Structure of some XIAP/BIRK3 inhibitors. AVPI peptide; some SMAC peptidomimetics (**a**); some conformationally constrained non-peptidic SMAC mimetics (**b**); Embelin, a monovalent SMAC mimetic (**c**); a bivalent SMAC mimetic (**d**)

higher affinity than the SMAC AVPI peptide. Indeed, functional studies have shown that SMAC protein is a much more efficient and potent antagonist than the AVPI peptide against XIAP protein containing the BIR2 and BIR3 domains in terms of relieving the XIAP-mediated inhibition of caspase-9, caspase-3, and caspase-7 activity. The SMAC AVPI-binding motif binds to both the BIR2 and BIR3 domains, although with a stronger affinity to BIR3. Thus, small molecules designed to have two “AVPI”-binding motifs may mimic the mode of action of SMAC protein to target XIAP and be capable of achieving very high binding affinities to XIAP by concurrently targeting both the BIR2 and BIR3 domains in the protein. The structure-based design of non-peptidic, bivalent SMAC mimetics based upon conformationally constrained monovalent SMAC mimetics has been reported. The study characterized in detail the interaction of both monovalent and bivalent SMAC mimetics with different XIAP protein constructs. As potential drug candidates, there are advantages and disadvantages associated with monovalent and bivalent SMAC mimetics (Fig. 2.7). Monovalent SMAC mimetics are less potent than their corresponding bivalent SMAC mimetics. However, monovalent SMAC mimetics, with a molecular weight of ~ 500 Da, possess many desirable pharmacological properties as potential drug candidates. Bivalent SMAC mimetics have been shown to be 100–1,000 times more potent than their monovalent counterparts and could potentially be far more efficacious. However, because bivalent SMAC mimetics have a molecular weight exceeding 1,000 Da, such compounds may be expected to have very low oral bioavailability and will have to be administered by other routes of administration, such as intravenous dosing,

which represents a potential disadvantage if the drug must be given to patients frequently [45].

In 2004, embelin, a natural compound (Fig. 2.7) from the Japanese *Ardisia* herb, was reported as a fairly potent, non-peptidic, small molecular weight, cell-permeable inhibitor that binds to the XIAP BIR3 domain. Embelin was identified through structure-based computer screening of the proprietary searchable three-dimensional structure database (TCM-3D) containing 8,221 small organic molecules with diverse chemical structures isolated from nearly 1,000 traditional Chinese medicinal herbs. Unlike most commercial databases, all of the compounds in the TCM-3D database are natural products derived from traditional medicinal herbs, which have been used for medicinal purposes in China and other countries for centuries. The extensive use of these traditional Chinese medicine recipes in humans has generated a great amount of data about their efficacy and safety [47].

2.7 p21-Ras

Ras proteins play a central role in cellular growth and differentiation. Ras signaling is tightly regulated by cycling between an active GTP-bound conformation (Ras-GTP) and an inactive GDP-bound state (Ras-GDP) (Fig. 2.8).

Ras has a slow intrinsic GTPase activity that is enhanced by GTPase-activating proteins (GAPs). These proteins greatly increase the rate of GTP hydrolysis and thereby act as negative regulators of Ras output. Neurofibromin also regulates Ras signaling pathways by accelerating the conversion of Ras-GTP to Ras-GDP. Oncogenic *RAS* alleles carry single-point mutations at the Gly12, Gly13, or Gln61 positions that greatly reduce its intrinsic GTPase activity and render these proteins resistant to GAPs. Oncogenic *RAS* mutations or the inactivation of *NF1* perturb Ras signaling by favoring the GTP-bound state (Fig. 2.8).

Growth factors induce cell growth, in part by activating guanine nucleotide-exchange factors (GEFs), such as Sos, GRF, and GRP, which bind Ras and stimulate nucleotide dissociation. Nucleotide exchange increases the percentage of Ras-GTP because the intracellular concentration of free GTP vastly exceeds that of GDP. Signaling terminates when Ras-GTP is hydrolyzed to Ras-GDP (Fig. 2.8).

Ras proteins regulate cell fates by transducing signals from the plasma membrane to the nucleus via a series of downstream effectors. Ras-GTP recruits Raf kinase to the membrane, where it initiates a kinase cascade involving MEK kinase and the Erk1 and Erk2 isoforms of mitogen-activated protein (MAP) kinase. The activation states of the phosphoinositol-3'-kinase (PI3K) and Rac/Rho pathways are also regulated by Ras-GTP in many cell types. The consequences of Ras activation are influenced by the cellular context and by cross-talk between its downstream effectors. Ras-GTP-mediated activation of the Raf/MEK/ERK kinase cascade stimulates proliferation in many cell types, and activation of the PI3K pathway has been shown to promote cellular survival. Ras also interacts with other downstream effectors, such as Ral-GDS.

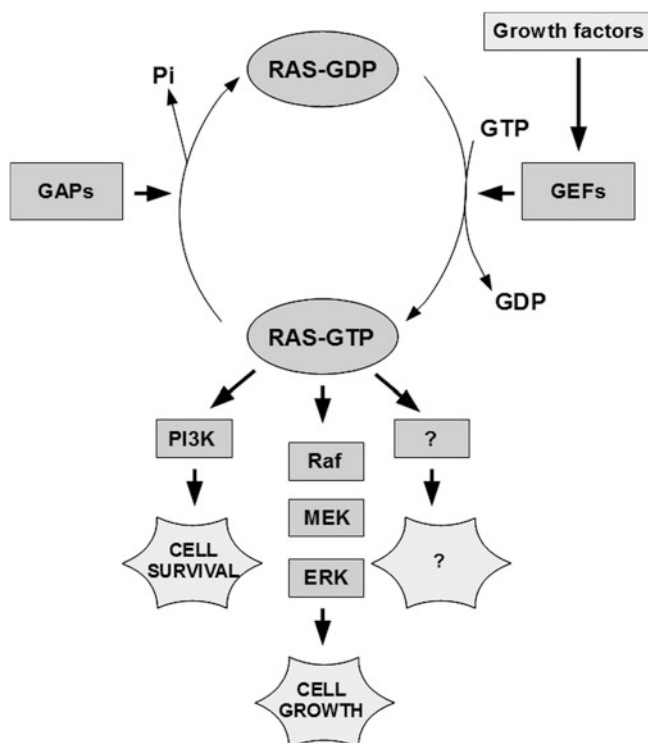


Fig. 2.8 Schematic representation of Ras cycling between the active *GTP-bound* conformation and the inactive *GDP-bound* state, the cascade effects of Ras activation and modulation by *GEFs* and *GAPs*

The posttranslational modification of Ras is initiated by the attachment of either a farnesyl or geranylgeranyl isoprenoid lipid to the cysteine residue of the carboxy-terminal CAAX box. These reactions are catalyzed by farnesyl and geranylgeranyltransferase, respectively. The last three amino acids (i.e., the -A-A-X) are then removed by a specific endoprotease, Rce1. The final step in Ras processing involves methylation of the carboxyl group by an endoplasmic reticulum-associated methyltransferase [48].

Recent studies suggest that the p21 product of the Ras oncogene may be an obligatory intermediate in transducing the growth factor signal. The activation of Ras may, therefore, activate the growth factor pathway without the need for either a growth factor or its receptor [49].

Functionally, all p21-Ras isotypes can be divided into three domains: (1) the NH₂-terminal portion, which contains the phosphate-binding and effector domains and has almost 100 % homology between isotypes; (2) the intermediate guanine recognition domain, which also has high homology; and (3) the COOH-terminal hypervariable region, which undergoes posttranslational modification, including farnesylation, leading to membrane localization. The crystal structure of inactive

and activated p21-Ras has also revealed two “switch” domains, in which the tertiary structure of the protein alternates between inactive and active states. The switch-1 domain corresponds to the effector domain in the NH2 terminus. The conformation of this domain is altered with activation, allowing the interaction of downstream effectors. The switch-11 domain is similar to an intermediate guanine nucleotide recognition domain, which assumes a different conformation that is dependent on the nucleotide exchange between GDP and GTP caused by the action of Ras-GEFs and Ras-GAPs. Data from recent mouse knockout studies have demonstrated that, despite their high degree of homology, p21-Ras isotypes do possess certain unique cellular functions [50].

Because efforts to identify and develop small-molecule inhibitors that directly target Ras have not been successful, a majority of past and ongoing efforts have targeted Ras indirectly, to modulate the functions of proteins that influence or mediate Ras oncogenesis.

The low micromolar-binding affinity of protein kinases for ATP, where potent nanomolar affinity ATP-competitive inhibitors have been developed (e.g., imatinib), has been a very successful avenue for anti-cancer drug development. In contrast, the low picomolar-binding affinity of small GTPases for GTP and millimolar cellular concentrations of GTP renders a similar strategy for Ras implausible. Thus, past and current efforts have focused on indirect approaches for the disruption of Ras function, that is, the inhibition of components that regulate Ras membrane association and the inhibition of downstream effector signaling.

One of these classes of proteins includes those that regulate Ras posttranslational processing, which is either signaled through the C-terminal CAAX tetrapeptide motif (farnesyltransferase, FTase; Rac-converting enzyme 1, Rce1; Isoprenylcysteine carboxyl methyltransferase, Icmt) or by protein kinase C α (PKC α)-dependent phosphorylation or ubiquitination.

Considerable past efforts centered on the development of FTase inhibitors (FTIs), with many identified and two remaining in clinical trial analyses (lonafarnib and tipifarnib, Fig. 2.9). The prenylation of K-Ras and N-Ras by a related enzyme, geranylgeranyltransferase-I, when farnesyltransferase activity is blocked by treatment with an FTI, proved to be the downfall of FTIs as effective Ras inhibitors [51, 52].

A second class of inhibitor of Ras membrane association is composed of two small molecules with farnesyl lipid groups (salirasib and TLN-4601, Fig. 2.9) that are proposed to compete with Ras for membrane-associated docking proteins for the Ras isoprenoid group. Efforts to target Ras effector signaling first centered on the Raf–MEK–ERK MAPK cascade. Small-molecule protein kinase inhibitors of MEK1/2, and later Raf, have been developed, with many inhibitors now under clinical evaluation. More recently, inhibitors of the p110 catalytic subunits of PI3K, AKT, and mTOR have entered clinical trials, and two mTOR inhibitors have been approved by the FDA for the treatment for renal cell cancers [52]. Moreover, Ras proteins are also regulated by multiple GEFs and GAPs. GTP-bound Ras interacts with catalytically diverse downstream effectors that possess

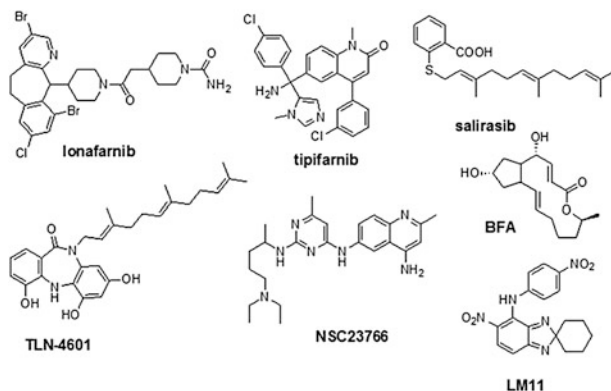


Fig. 2.9 Structures of some FTase inhibitors (lonafarnib, tipifarnib), inhibitors of Ras membrane association (salirasib, TLN-4601), and GEF inhibitors (BFA, LM11, NSC23766)

Ras-binding (RBD) or Ras association (RA) domains. Some of these interactions are restricted to specific Ras isoforms [52].

Brefeldin A (BFA, Fig. 2.9) is a natural product isolated from the fungus *Eupenicillium brefeldianum* and is the first known inhibitor of a GEF. The extensive analysis of the mechanism of action of BFA led to the general concept of “interfacial inhibition,” which refers to inhibitors that act by stabilizing protein complexes and target regions in or near interfaces. Some inhibitors of natural origin that are already used in the clinic have been recognized as interfacial inhibitors, such as the anti-cancer drugs vinblastine or camptothecin. LM11 (Fig. 2.9) was discovered by an in silico screen and was shown to target an interfacial depression at the surface of the complex between Arf1–GDP and BFA-insensitive GEFs, such as ARNO, and to block ARNO-dependent cellular migration. A few other promising examples of cell-active small-molecule ArfGEF inhibitors have been selected by in vitro and phenotypic screens. These studies demonstrate that despite the high levels of homology that are found within a given GEF family, specific GEF inhibitors can be developed. Therefore, the specific flexibility and conformational changes that characterize small GTPase–GEF complexes are likely to be advantageous to drug development, notably for interfacial inhibitors. However, the design of high-throughput biochemical assays to effectively screen for such inhibitors remains a challenge. Inhibitors that target specific RhoGEFs have been discovered by high-throughput screens.

Another related example of a way to target GTPase activity is through targeting the surface of GTPases that is required for GEF activation. The small-molecule NSC23766 (Fig. 2.9) was discovered through computational screening of the surface of Rac1, which is known to interact with GEFs. This compound was found to inhibit the activation of Rac1 by the Rac-specific GEFs, Trio, and Tiam1.

There is limited but promising evidence that it may be possible to develop small-molecule modulators of Ras superfamily GAPs. HTS identified small-molecule inhibitors of RGS domains, which are GAPs for heterotrimeric G proteins.

Despite their low structural homology to RasGAPs, they share a similar enzymatic transition state, suggesting that this could be a starting point for the design of Ras superfamily GAP inhibitors. The Rac-selective chimaerins (CHN), one class of RhoGAPs, possesses C1 zinc finger domains that bind diacylglycerol, a cofactor for their activity. Therefore, small molecules that bind C1 domains may activate their GAP activities, causing the downregulation of Rac GTPase activity. While such a therapeutic approach will be complicated by the existence of other proteins with C1 domains (e.g., RasGRP), there is evidence that C1-binding molecules can have some degree of selectivity for a subset of C1-containing proteins. This approach may be a therapeutic option for cancers in which there is RacGEF-mediated activation of Rac [52].

Another approach to antagonize Ras functions *in vivo* is the inhibition of Ras expression by anti-sense RNA-based technology, the use of anti-Ras ribozymes (hammerhead models), or the application of anti-Ras retroviral therapy [53].

2.8 Human Thymidylate Synthase

Human thymidylate synthase (hTS) is an enzyme that plays a key role in DNA synthesis and is a target for several clinically important anti-cancer drugs. Inhibitors of hTS are widely used in chemotherapy; the best known inhibitors are raltitrexed, pemetrexed, and 5-fluorouracil [54]. However, their use is associated with drug resistance. Therefore, compounds with different inhibitory mechanisms are required to combat resistance. Some peptides have been designed to specifically target the protein–protein interface in hTS, which is a dimeric protein composed of two identical polypeptide chains. The peptides stabilize the inactive form of the enzyme and inhibit cell growth in drug-sensitive and drug-resistant cancer cell lines.

TS (EC: 2.1.1.45) catalyzes the reductive methylation of 2'-deoxyuridine-5'-monophosphate (dUMP) to 2'-deoxythymidine-5'-monophosphate (dTMP), which is assisted by the cofactor N⁵, N¹⁰-methylene tetrahydrofolate (MTHF) [55–57]. Because TS represents the only synthetic source of dTMP in cells, it is a major target for the design of chemotherapeutic agents [58].

In addition to its catalytic role, hTS has also been shown to regulate protein synthesis by interacting with its own messenger RNA as well as those of several other proteins involved in the cell cycle [59]. The regulatory function of hTS as an RNA-binding protein has been shown to be maximal when the protein is not bound by ligands. This observation, together with the observation that cancer cells resistant to anti-hTS drugs, has increased levels of hTS, led to the hypothesis that the overexpression of hTS might be correlated with the loss of RNA regulatory capacity when the protein is bound to its inhibitors [60]. In the case of ovarian cancer, for example, access to therapy has been limited by the cross-resistance of platinum drugs with classical drugs that target the metabolic pathway that uses folic acid and its derivatives as substrates and/or cofactors. The same observation

may be valid for other cancer types, such as colorectal cancer, in which TS inhibitors are widely used. Although the overexpression of hTS has been observed in platinum-sensitive cells, this effect is even more pronounced in platinum-resistant ones. Therefore, it is important to identify hTS inhibitors that act through new mechanisms that do not alter RNA regulation or increase protein levels [61]. When Schiffer and coworkers [62] first crystallized the unliganded form of hTS, they found that the active site loop (residues 181–197) containing the catalytic cysteine (Cys195) was twisted approximately 180 degrees compared to the corresponding loop conformation in the liganded hTS. Because in unliganded hTS, Cys195 is outside the active site, the enzyme must be inactive (Fig. 2.10). The authors suggested that the inactive conformation might serve to protect the catalytic cysteine from cellular modification. Three phosphate/sulfate ions were observed to be bound near the active site, suggesting that inactive hTS can bind to TS mRNA, thereby repressing TS protein synthesis. In addition, the disordered small domain (residues 107–128) of the inactive hTS likely increases its proneness to cellular degradation, further reducing cellular TS levels. They demonstrated through fluorescence studies that there is an equilibrium between the active and inactive states; phosphate ions were shown to shift the equilibrium toward the inactive state, and the binding of dUMP causes a shift toward the active state [63]. The R163K mutant, which stabilizes the active conformer, is at least 33 % more active than wild-type hTS, suggesting that at least 1/3 of hTS exists in the inactive state [64]. On the basis of these data, some diphosphonic acids have been proposed as inhibitors of hTS. The most active one is 1, 3-propanediphosphonic acid (PDPA), which binds at the position where the phosphate group of the substrate dUMP binds. However, the mechanism of inhibition of these compounds has not yet been biochemically and mechanistically demonstrated. Furthermore, PDPA has no cellular activity, and thus its potential as a drug is limited.

On the basis of structural considerations, PDPA was discovered as an allosteric inhibitor of hTS. It has been proposed that PDPA acts by stabilizing an inactive conformer of loop 181–197. Kinetic studies showed that PDPA is a mixed (non-competitive) inhibitor versus dUMP. In contrast, PDPA is an uncompetitive inhibitor versus MTHF at concentrations lower than 0.25 μM , but at PDPA concentrations higher than 1 μM , the inhibition is non-competitive, as expected. At the concentrations corresponding to uncompetitive inhibition, PDPA shows positive cooperativity with an antifolate inhibitor, ZD9331, which binds to the active conformer. PDPA binding leads to the formation of hTS tetramers. These data are consistent with a model in which hTS preferentially exists as an asymmetric dimer with one subunit in the active conformation of loop 181–197 and the other in the inactive conformation.

A new area of investigation is the discovery of several peptides [65], with sequences from the protein–protein interface of the hTS dimer that inhibit hTS by a mechanism that involves selective binding to a novel binding site at the dimer interface of the inactive form of the enzyme. The peptides were designed to contain a sequence that is complementary to the monomer surface, and therefore, these peptide inhibitors are protein–protein interaction modulators. The

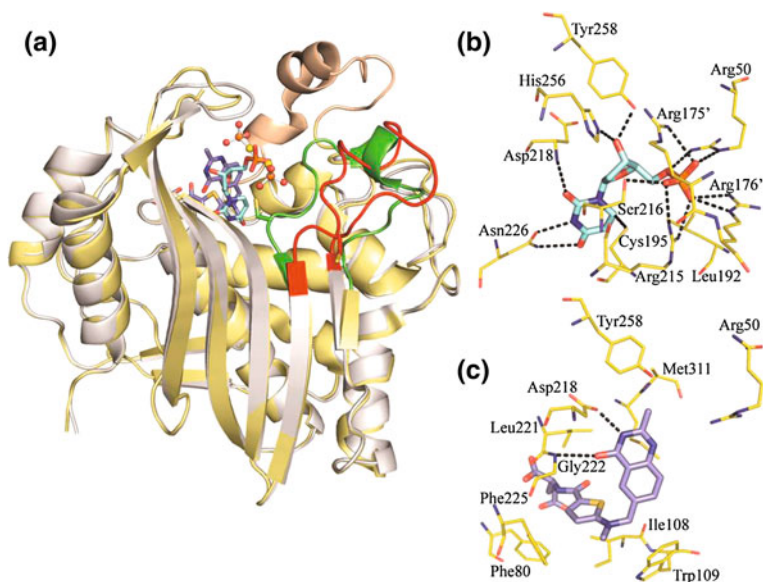


Fig. 2.10 **a** Cartoon representation of the superimposed monomeric subunits (from the crystallized dimers) of human thymidylate synthase in active (PDB 1HVY) (yellow) and inactive (PDB 2ONB) (gray) conformations. The active conformation of the active site loop is shown in green and the inactive conformation in red. The catalytic cysteine, C195, is highlighted with a stick representation on the loops. The small domain visible in the active crystal structure is shown in brown. dUMP (cyan sticks) and a folate analog, raltitrexed (dark blue sticks), are present in the active site, whereas PDPA (ball-and-stick representation) is located in an allosteric position. **b** Interactions of dUMP with the protein. Dashed lines represent direct hydrogen bonds between amino acid residues and the ligand, the solid line between Cys195 and dUMP represents a covalent bond. **c** Interactions of the folate analog, raltitrexed, with the protein

mechanism of inhibition has been demonstrated through a combination of experimental and computational approaches. Kinetic analyses, isothermal titration calorimetry, fluorescence spectroscopy, X-ray diffraction, and modeling studies show that this mechanism differs from those of the protein–protein interface inhibitors reported to date [2] because it involves the stabilization of an inactive form of the catalytic protein. The kinetic analysis suggests that these peptides behave as mixed-type inhibitors. The X-ray crystal structure of the hTS–LR peptide complex unambiguously shows that LR binds at the monomer–monomer interface and that the two monomers of the enzyme must move apart for the peptide to bind properly. However, Foerster resonance energy transfer (FRET) experiments show that the hTS monomers do not dissociate upon peptide binding. Calorimetric results indicated that the peptides only bind to one of the isoforms of the enzyme and that no binding is observed when the protein is saturated with the substrate dUMP, which is known to convert the protein to its active form. On the basis of these data, an inhibition model characterized by the stabilization of the inactive form of the enzyme by peptide binding has been proposed. This model is consistent with physical and biochemical observations.

Unlike the existing drugs that target hTS, these peptides inhibit intracellular hTS as well as cell growth without resulting in overexpression of hTS when administered to ovarian cancer cells. The connection between the stabilization of the inactive form of hTS by the peptides and the cellular effects remains to be fully explored. Further steps will require the optimization of the compounds via the synthesis of peptidomimetics and a detailed analysis of their cellular mechanism of action. The study demonstrates a mechanism in which a multifunctional homodimeric protein is inhibited through the binding of a ligand to the dimer interface of the inactive form of the enzyme, resulting in its stabilization. The concepts revealed here can be exploited to provide new strategies for the development of drugs for combating diseases such as ovarian cancer.

2.9 Natural Compounds as PPI Inhibitors

Several screening studies for the evaluation of the capacity of natural products to inhibit PPIs have been described in the literature. Natural product chemistry offers several advantages in drug discovery, mainly due to the great variety of molecular sizes and chemical structures provided by secondary metabolites isolated from plant species, fungi, microorganisms, marine invertebrates, algal species, and sponges. In particular, targeting PPIs with small natural compounds has been demonstrated to be a valuable application in the scope of anticancer drug discovery, as shown by several screening studies reported in the literature.

A first example is represented by a screen of approximately 7,000 purified natural compounds at 10 μM using high-throughput ELISA to identify molecules that inhibit the association between T-cell factor 4 (Tcf4) and β -catenin, which represents a rational and feasible target for the development of drugs against colorectal cancer [66]. Eight compounds resulted in a reproducible dose-dependent inhibition of the Tcf4/ β -catenin interaction, with $\text{IC}_{50} < 10 \mu\text{M}$ [66]. The group of active compounds had varying chemical structures, although some of them share polyhydroxylated planar features. Three compounds were isolated from fungal organisms, whereas three originated in actinomycete strains. One of the active molecules was isolated from a marine sponge, whereas the origins of the remainder were not specified. Further assays carried out to demonstrate that the isolated compounds work as predicted indicated that two fungal derivatives, named PKF115-584 and CGP049090 (Fig. 2.11), were able to disrupt the Tcf4/ β -catenin complex in vitro and to inhibit colon cancer proliferation [66]. These antagonists of the Tcf4/ β -catenin complex were also able to inhibit the proliferation of adrenocortical carcinoma [67] and hepatocellular carcinoma [68] cell lines.

Another example is the study of Tsukamoto et al. [69], who described the inhibition of the p53/HDM2 interaction using a simple unsaturated dicarboxylic acid named (–)-hexylitaconic acid (Fig. 2.11), which was isolated from a culture broth of the marine-derived fungus *Arthrinium* sp. HDM2 (human double minute 2) or MDM2 (mouse double minute 2) is a ubiquitin ligase that induces the

degradation of p53, a tumor suppressor protein [70]. Targeting HDM2 or MDM2 is a useful tool to activate p53 and induce growth arrest and apoptosis in cancer cells [70]. The isolated natural compound was able to inhibit the p53/HDM2 interaction, and the IC_{50} value was found to be 50 $\mu\text{g/mL}$ [69], as determined by ELISA. A more functionalized and complex natural product with the same activity is chlorofusin [71]. Chlorofusin is a highly modified cyclic peptide isolated from a microfungus of the genus *Fusarium*, which was demonstrated to inhibit the p53/MDM2 interaction with an IC_{50} value of 4.6 μM [71].

A further example of the capacity of natural compounds to inhibit PPIs is provided by Hashimoto et al. [72]. The authors developed a HTS system using an in vitro protein fragment complementation assay (PCA) to test the possible inhibition of the interaction between T-cell factor 7 (TCF7) and β -catenin, proteasome assembling chaperone (PAC)1 and PAC2, and the self-association of PAC3 homodimer, each of which play an important role in the growth of cancer cells, from 123,599 samples in a natural product library. A fungal metabolite named

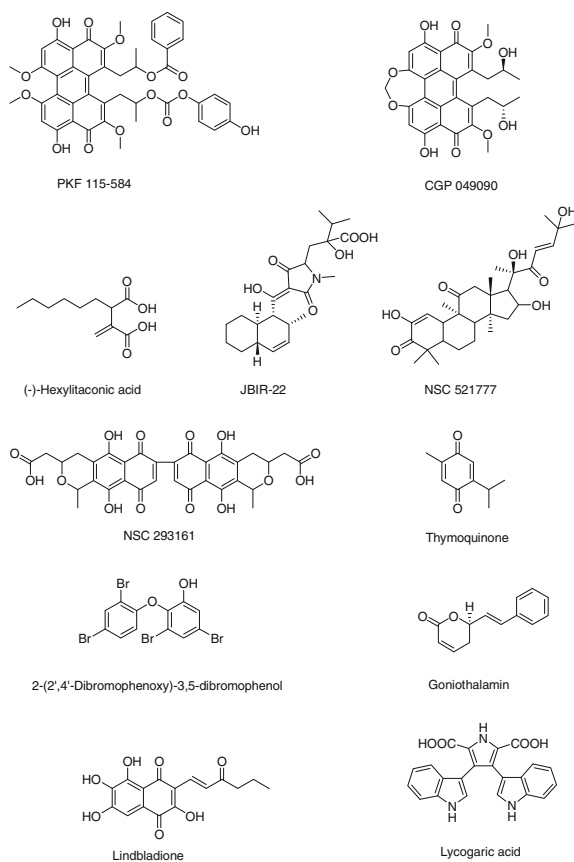


Fig. 2.11 Chemical structures of natural compounds able to inhibit PPIs

TB1, whose structure was not shown, was found capable of strongly and specifically inhibiting PAC3 homodimerization, with an IC_{50} value of $0.020\ \mu\text{M}$ and was recognized as an invaluable lead compound for the development of new anticancer drugs. Another compound named JBIR-22 (Fig. 2.11) showed a specific inhibitory activity on PAC3 homodimerization, with an IC_{50} value of $0.2\ \mu\text{M}$ [72]. This compound is a fungal metabolite isolated from *Verticillus* sp., whose structure was correctly clarified in a later study [73] and was revealed to be a new equisetin analogue on the basis of extensive NMR and MS analyses. Docking studies of this secondary metabolite with the X-ray structure of PAC3 revealed that it can bind to the active site of the PPI required for PAC3 homodimerization. In the same study [73], the cytotoxic activity of the isolated compound in the human cervical carcinoma cancer cell line (HeLa) was evaluated. The tested secondary metabolite displayed an IC_{50} value of $68\ \mu\text{M}$ at 120 h, showing no effect at the same concentration after 48 h [73]. These results suggest that it may inhibit the generation of proteasome components.

In another study, a virtual screening of molecules present in the National cancer institute (NCI) molecular database, known as “NCI-diversity,” which features approximately 2,000 anticancer agents based on fairly different chemical scaffolds, identified ten compounds, two of which were of natural origin, as inhibitors of stem cell factor (SCF) [74]. SCF is an endogenous growth factor involved in the hematopoietic cell proliferation and differentiation [74]. It also plays a crucial role in the development of melanoma and several intestinal cancers [74]. Similar to other growth factors, SCF dimerization is a necessary step in the activation of its natural tyrosine kinase receptor, c-kit [74]. The SCF/c-kit interaction leads to the first step of a biochemical cascade responsible for several effects, including cell proliferation. The first identified natural compound, named NSC 521777, which passes Lipinski’s rules, is a cucurbitacin (Fig. 2.11), and it has been reported as an active anticancer compound involved in the tyrosine kinase signaling pathway [74]. The second one, named NSC 293161, is the natural antibiotic actinorhodin (Fig. 2.11) [74] and has been shown to possess a bis anthraquinonic structure. Biological assays of the top scored compounds are in progress.

Another example of PPI inhibition by small natural compounds has been described by Reindl et al. [75]. In this work, a screen of diverse chemical libraries, consisting of 22,461 small molecules, was carried out using a fluorescence polarization assay to identify compounds that could interfere with Polo-like kinase 1 (Plk1). Plk1 is a regulator of multiple stages of mitotic progression, and it is over-expressed in many types of human cancers. Its inhibition by small molecules has been demonstrated to induce mitotic arrest and apoptosis in cancer cells, both in vitro and in vivo [75]. Usually, inhibitors of Plk1 target the conserved ATP-binding site. The authors demonstrated that Plk1 can alternatively be targeted by small molecules that inhibit the function of the polo-box domain (PBD), a recently discovered protein domain that mediates the intracellular localization of Plk1 [75]. In particular, the natural compound thymoquinone (Fig. 2.11), the bioactive constituent of the volatile oil of black seed (*Nigella sativa*) [76], represents the first non-peptidic inhibitors of the PPI mediated by Plk1/PBD (apparent $IC_{50} = 1.14 \pm 0.04\ \mu\text{M}$)

[75]. This compound and its synthetic derivative inhibit the function of Plk1/PBD in vitro and cause Plk1 mislocalization, defective chromosome congression, mitotic arrest, and apoptosis in HeLa cancer cells [75]. In this way, the anticancer activity of thymoquinone has been demonstrated, making it one of the few natural compounds found capable of inhibiting a PPI.

Another important target in the field of PPI inhibition is represented by the interaction between the Mcl-1 and Bak proteins [77]. The overexpression of the Mcl-1 protein in cancer cells results in the sequestration of Bak, a key component in the regulation of normal cell apoptosis. An investigation on the ability of marine-derived small natural products to disrupt the interaction between Mcl-1 and Bak led to the isolation of 13 oxy-polyhalogenated diphenyl ethers (O-PHDEs) from the marine sponges *Dysidea granulosa* and *Dysidea herbacea*. These molecules exhibited an interesting activity in a preliminary FRET assay (IC_{50} values of 4.1 and 2.1 $\mu\text{g/mL}$, respectively) [77]. The isolated secondary metabolites derive from an association between the above-cited sponges and a symbiotic cyanobacterium, named *Oscillatoria spongelliae* [77]. Unfortunately, the bioassay-guided fractionation did not lead to the isolation of pure constituents with a higher activity than the crude extracts. In fact, only three of these compounds exhibited significant IC_{50} values $<10 \mu\text{g/mL}$, with only one compound, named 2-(2',4'-dibromophenoxy)-3,5-dibromophenol (Fig. 2.11), showing an IC_{50} value of 2.1 $\mu\text{g/mL}$ [77]. A synergistic effect, due to the combination of O-PHDEs, was believed to occur in the crude extracts.

Small molecules of natural origin have also demonstrated to be capable of controlling protein transport by interfering with PPI. An example is represented by the transport of proteins from the nucleus to the cytoplasm by the exportin CRM1, which recognizes cargo proteins through a leucine-rich nuclear export signal (NES) [78]. By analogy with a truncated analog of the anguinomycins, which are potent anticancer agents active in the picomolar range that belong to the leptomycin family of natural products, goniothalamine (Fig. 2.11) was discovered [79]. This compound is a member of a class of styryl lactones isolated from plants of the genus *Goniothalamus*. Goniothalamine has been reported to induce cytotoxicity in breast cancer cells, with an IC_{50} value of ca. 1.5 μM , causing growth arrest and apoptosis [79]. These effects have been attributed to the inhibition of nuclear export by this natural compound at concentrations above 500 nM, as demonstrated by the immunostaining of Rio2 kinase in HeLa cells [79], based on the disruption of the PPI between CRM1 and cargo proteins [79]. With the discovery of the mechanism of action of this natural product, the search for structurally simpler leptomycin analogs useful for anticancer therapy is of great interest.

Novel inhibitors of PPIs may also be applicable in regenerative medicine for the treatment for neuronal diseases, especially for the generation of new neurons after stroke [80]. To this end, a rapid in vitro HTS for identifying natural compounds able to inhibit Hes1 dimer formation has been described [80]. The inhibition of Hes1 dimerization results in the acceleration of basic-helix-loop-helix (bHLH) activator transcription, which may promote the differentiation of neural stem cells (NSCs) into neurons. The developed assay was based on the use of fluorophore-

labeled Hes1 and Hes1 immobilized on microplates [79]. In the presence of an inhibitor, the level of Hes1 dimerization was reduced, as detected by a corresponding reduction in fluorescence. With this system, six compounds able to inhibit Hes1 dimerization were identified from a library of natural products composed by secondary metabolites isolated by the authors [80]. Three of the identified active compounds are natural products from myxomycetes; one was isolated from *Jasminum grandiflorum* and two from *Saraca asoca*. The most active compound was lindbladione ($IC_{50} = 4.1 \mu M$) (Fig. 2.11), which was isolated from the myxomycetes *Lindbladia ubulina*. Furthermore, using a cell-based reporter gene assay, it has been demonstrated that two of the identified compounds, lindbladione and lycogaric acid (Fig. 2.11), the latter of which was isolated from the myxomycetes *Lycogala epidendrum*, inhibited the Hes1-mediated suppression of transcription in C3H10T1/2 cells.

2.10 Perspectives

A few protein–protein interaction inhibitors are nowadays entering in clinical trials. The first MDM2 inhibitor that entered clinical development is RG7112 from Hoffmann-La Roche (clinicaltrials.gov identifiers: NCT01164033, NCT01143740, NCT00623870, and NCT00559533). Four phase I clinical trials have been conducted to date in patients with advanced solid tumors, hematologic neoplasms, or liposarcomas prior to debulking surgery. Preliminary clinical data indicated that RG7112 appears to be well tolerated in patients and shows initial evidence of clinical activity and a mechanism of action consistent with targeting of the MDM2–p53 interaction. Other candidates are along the same line therefore it becomes clear that the possibility to develop protein–protein interaction inhibitors that are effective anticancer drugs with the predicted mechanism of action is a concrete perspective. The monitoring of the drug development process can be implemented assisting each step from the computational design to the lead optimization, cellular pharmacology, and animal testing with dedicated strategies focused on PPIs inhibitors mechanism. The monitoring process includes analytical tools and pharmacodynamic biomarkers that will ensure an higher and accelerated success rate.

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