# Specificity in the Interaction of Natural Products with their Target Proteins- A Biochemical and Structural Insight

P. Venkatraman*

*Advanced Centre for Treatment Research and Education in Cancer, Tata Memorial Centre, KS 137, Sector 22, Kharghar, Navi Mumbai, Maharashtra 410210 India*

**Abstract:** Natural products are an abundant source of anti cancer agents. They act as cytotoxic drugs, and inhibitors of apoptosis, transcription, cell proliferation and angiogenesis. While pathways targeted by natural products have been well studied, there is paucity of information about the *in vivo* molecular target/s of these compounds. This review summarizes some of the natural compounds for which the molecular targets, mechanism of action and structural basis of specificity have been well documented. These examples illustrate that 'off target' binding can be explained on the basis of diversity inherent to biomolecular interactions. There is enough evidence to suggest that natural compounds are potent and versatile warheads that can be optimized for a multi targeted therapeutic intervention in cancer.

**Keywords:** Natural products, molecular target, specificity, mechanism, structure, cancer.

## INTRODUCTION

Over centuries, plant and microbial derived natural compounds have been successfully used in the treatment of many diseases including cancer, the most complicated, multi faceted, highly heterogeneous and hard to combat disease. Approximately 47% of the drugs currently used in the treatment of cancer are either derived directly from nature, or borrowed and modified [1]. In a wide variety of carcinogen induced animal models and in cells derived from tumors, natural products and their derivatives affect multiple pathways such as cell proliferation, signaling, transcription and apoptosis [2-10]. The mechanism of action of these compounds seems to be as complex as the molecular manifestation of the disease itself and the most effective compounds as well as the drugs in clinical use affect these pathways either directly or indirectly. For example, camptothecin is an inhibitor of topoisomerase I [11] while doxorubicin, a microbial product, in addition to intercalating to DNA, inhibits DNA-topoisomerase II [12, 13]. In both the cases the inhibitors act by stabilizing the DNA-topoisomerase complex, prevent unwinding of the DNA required for replication, and induce irreparable breaks in the DNA all of which eventually lead to cell death. Both imatinib (gleevec), a potent inhibitor of the kinase activity of the oncoprotein BCR-ABL [14] and gefitinib, which inhibits the catalytic activity of epidermal growth factor receptor (EGFR) [15], prevent signaling leading to cell death. Vinblastine, taxol and vincistrine affect tubulin polymerization [16] causing cell cycle arrest, which leads to activation of the apoptotic machinery.

Several years of research has led to the identification of many more potential molecular targets amenable for manipulation in cancer cells [17, 18]. For example, cyclins that regulate the cell cycle [2], proteins involved in DNA repair, proteins directly regulating the apoptotic pathway like Bcl-2 [19], those that promote angiogenesis like vascular endothelial growth factor and its receptor (VEGF, VEGFR respectively) [20], those involved in metastasis like the matrix metalloproteases, (MMP) [20], tumor suppressor proteins like p53 [21], the ubiquitous ATP dependent protease called the proteasome, and the molecular chaperone Hsp90, are some of the 'desired' molecular targets [22, 23]. A handful of compounds that can target these molecules with a promise for clinical use are known and a couple of them are already in the market (e.g., velcade against proteasomes and geldanamycin against Hsp90). With their enviable number and diversity of functional groups, plant and microbial derived compounds may actually be nature's solution for a targeted approach in drug discovery. However, the very versatility and diversity of these compounds seems to be their bane – the fact that a single compound affects multiple pathways [7, 24-27] makes it likely that *in vivo* such compounds could bind to molecules indiscriminately with unpredictable outcome. This has created a sense of ambiguity in accepting the pharmacological effect of these compounds. But even with drugs that are already in use for treatment of various types of cancers, the actual *in vivo* target is not unambiguously defined. And in instances where the identity is fairly well established, the exact consequence of such specific interaction seems to be rather speculative and subject to further intense research [28-30]. In citing the example of gleevec which is a highly selective inhibitor of the oncoprotein BCR-ABL, a tyrosine kinase, Kamb *et al.*, point out that BCR-ABL was proven to the actual *in vivo* target only when drug resistance mutations of the kinase began to surface [31]! Results from studies with gleevec were for a long time treated as an exception but, over the years, it has become evident that the specificity and selectivity of gleevec may have been over rated. Nevertheless there are several compelling reasons why it is desirable to know the identity of the molecular target of a potential drug. For example, effect of the drug in changing

*Address correspondence to this author at the Advanced Centre for Treatment Research and Education in Cancer, Tata Memorial Centre, KS 137, Sector 22, Kharghar, Navi Mumbai, Maharashtra 410210 India; Tel: 022-27405091; Fax: 022-27405085; E-mail vprasanna@actrec.gov.in

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the phenotype of the disease is more convincing when the molecular target is known. This helps in further design of a compound with more favorable properties and low off target effect and toxicity. If the molecular target is known, then its likely role in the context of a pathway may help in predicting the type of cancer to be treated, the disease status and in choosing the right patient population that would respond to the treatment. It may even be possible to design strategies to overcome drug resistance and probably prevent relapse. If there are multiple targets, modified versions of the compound with differential affinity can be prepared to obtain a synergistic effect.

With this back-ground, the review discusses some of the molecular targets of drugs based on natural products, and derivatives or mimics of natural products which are already in use for the treatment of cancer as well as those in clinical trials. The emphasis is on the mechanism of interaction between the drug and the intended molecular target, the structural and chemical basis of selectivity, the identity of the 'off target' candidates. Examples discussed include drugs that are selective for a particular protein conformation, drugs that interfere with protein-protein interactions and drugs that contain a functional group, the selectivity of which is controlled by the nature of the functional group and the type of the scaffold present. These examples are put forth to emphasize the point that more research is necessary in this direction despite the fact that the results obtained may sometimes be confusing or inconclusive. The summary also illustrates the point that a lot is to be gained by accepting the fact these natural products are multi functional in nature and this functionality has to be exploited in the drug discovery program aimed at targeting multiple pathways [10].

## 1. DRUGS SELECTIVE TOWARDS A SPECIFIC PROTEIN CONFORMATION

### 1.1. Gleevec

As mentioned earlier, the story of gleevec in the treatment of chronic myelogenous leukemia (CML) and the selectivity of the compound for BCR-ABL the oncogenic kinase is almost legendary and has not been seen with any other molecule so far. CML is typically characterized by the presence of an altered chromosome called the Philadelphia chromosome arising from the reciprocal fusion of chromosomes 9 and 22. The resultant is a new cancer specific protein product called BCR-ABL, which is an unregulated constitutively active kinase, the activity of which leads to uncontrolled cell proliferation. Efforts to identify a unique inhibitor of this kinase led to the discovery of imatinib, popularly known as gleevec, which was indeed highly selective to BCR-ABL (other major targets being KIT and PDGFR). Related kinases like-c-Src and Hck showed very weak binding to gleevec with $IC_{50}$ values differing by several orders of magnitude (Abl11±3, c-Src > 10,000 and Hck 5600 nmol/L) [32]. To explain the high selectivity of gleevec for Abl, crystal structures of the complex were solved at atomic resolution. Despite the remarkably low affinity of c-Src kinase for gleevec, the complex did crystallize. Comparison of the structures led to the interpretation that gleevec specifically binds to an inactive form of Abl and stabilizes this conformation, preventing it from binding to ATP. c-Src apparently incurs an immense thermodynamic penalty in binding to gleevec [33]. Several amino acid replacements were attempted to convert the c-Src to an Abl like conformation, but these attempts were unsuccessful. Such detailed structural characterization and mutational studies did not provide a completely satisfactory solution to the selectivity of gleevec for Abl over c-Src. However such iterative experiments were the basis for the next generation inhibitors of Abl and its mutant [34].

Recently, based on affinity chromatography and high throughput mass spectrometry, other cellular targets that could bind to gleevec have been identified. Surprisingly, quinone reductase 2 or NQO2-a non-kinase (Flavin Adenine Dinucleotide (FAD) and not Adenosine Triphosphate (ATP) is the ligand), was found to bind gleevec-with an $IC_{50}$ value of 82 nM, very close to that of Abl (~11nM). A key to this recognition seems to be the ability of NQO2 to adopt a conformation similar to that of Abl in presence of gleevec [35]. However NQO1-a close homolog of NQO2, does not bind to gleevec. Similarly NQO2 does not bind to NADH or NADPH, the reducing equivalents used by NQO1 as well as other NQO1 specific inhibitors [36].

This observation has opened up new questions regarding the biological relevance of the inhibition of NQO2 by gleevec. The normal physiological role played by NQO2 is unclear. However, RNAi knock down of NQO2 in BCR-ABL positive human myelogenous K562 cells, resulted in the reduction of cell proliferation [37] indicating that NQO2 has a definitive role to play in cells with this oncoprotein back-ground.

### 1.2. Prediction of Additional Targets of Gleevec and Resveratrol

Before the remarkable observation that gleevec can bind to NQO2-a non kinase, NQO2 has been well characterized as a target of the natural product resveratrol, isolated from grapes. Similar to the effect seen with the RNAi knockdown of NQO2, treatment of K562 cells with resveratrol also resulted in reduced proliferation [37, 38]. Attempts to find the relevant molecular target of resveratrol from the cytoplasmic fraction of K562 cells using an affinity matrix led to the identification of NQO2, which remained bound to the column even after stringent high salt washes [39]. Because of the observation that NQO2 a resveratrol target also bound to gleevec, we looked into the literature for other molecular targets of resveratrol. Ribonucleotide reductase (RNR) [38], F0F1- ATPase [40], cyclin dependent kinase (CDK), CDK7 [41] and DNA polymerase [27] are the probable candidates. Among these RNR is an enzyme responsible for *de novo* synthesis of deoxy ribonucleotides and vital for DNA replication and cell proliferation [42]. Some of the inhibitors of this enzyme are in clinical use and in clinical trials for the treatment of cancer [43]. Resveratrol was shown to inhibit purified RNR *in vitro* as well as decrease the rate of DNA synthesis in K-562 cells. The mechanism of inhibition was attributed to the ability of resveratrol to destroy the tyrosyl radical mandatory for the catalysis of RNR. [38]. Whether this effect is mediated by the direct binding of resveratrol to RNR remains to be demonstrated.

As mentioned before, NQO2 was identified in a global chemical approach using an affinity matrix of gleevec. In the same study, RNR was identified as one of the proteins which bound the affinity column. RNR did not pass the high stringency filter imposed for positive identification of proteins during database searches of the mass spectrometric data. However three specific peptides of RNR were experimentally identified (raw data supplemental Table V [44]).

The above observations prompted us to ask whether we can identify a binding site for resveratrol and gleevec in ribonucelotide reductase. Activity of RNR is under the feedback control of the products which regulate the levels of deoxy nucleotide triphosphate (dNTP) pools in the cells necessary for DNA synthesis and repair [45, 46]. This allosteric regulation involves complicated mechanisms and is not fully understood [46]. The enzyme functions in different oligomeric forms in different species but the general pattern of specificity of regulation seems to be common [42, 46]. There are two allosteric sites in the enzyme-the specificity site and the general allosteric site. Reaction is dictated by the type of nucleotide/deoxy nucleotide triphosphates (dNTP) occupying the specificity or the effector site. ATP and dATP stimulate reduction of cytosine and uridine ribonucleotides, dTTP and CTP stimulate reduction of guanosine ribonucleotides, while dGTP stimulates the reduction of adenosine ribonucleotides [45, 46]. One of the most crucial conformational changes observed in the allosteric regulation of RNR is the stabilization of loop 1 and loop 2 present at the dimer interface. In response to the occupancy at the effector pocket, loop 2 moves to open up the active site [46].

We used the crystal structure of RNR from humans as the template (PDB 2WGH-yet to be cited in Pubmed) and tried to model the binding of resveratrol and gleevec using the stand alone version of auto dock [47]. Bound ligands were removed prior to docking. Two putative sites were identified for resveratrol and gleevec and the binding pocket involving the dimer interface of RNR will be described here. As seen in Fig. (**1**), both resveratrol Fig. (**1A**) and gleevec Fig. (**1B**) occupy a pocket within chain A and make several contacts with the protein. Resveratrol binds along the length of the protein, and gleevec is bound in a fairly extended conformation and takes the contour of chain A. Both resveratrol and gleevec make contact with loop 1 and loop 2 residues,. As explained above, both loop 1 and loop 2 are important sensors of effector binding and become stabilized upon nucleotide occupancy. If resveratrol and gleevec do indeed bind to RNR as predicted by our model, the binding is likely to prevent loop 2 dynamics-a key event necessary for activity. This would prevent turnover of the enzyme and lock the enzyme in an inactive conformation. Conformational freezing of the protein seems to be a predominant mechanism by which some of the natural products inhibit the function of the cognate molecular target (see gleevec-Abl interaction above and flavopiridol-cyclin interactions as discussed below).

Although binding of resveratrol and gleevec to RNR has not been tested directly, based on the above lines of reasoning and our modeling studies indicative of a binding pocket for both, it would be interesting to test the binding of resveratrol and gleevec to RNR using purified proteins. Furthermore resveratrol may be tested in combination with gleevec for their probable synergy in the anti-proliferative effect on K562 cells.

### 1.3. Flavopiridol

Flavopiridol has its origin in a natural product, rohitukine. Several Phase I and Phase II clinical trials are being performed with flavopiridol, either alone or in combination with other anticancer agents [48]. Flavopiridol directly binds to many target proteins which are related in structure but different in their function. It is a broad specificity CDK inhibitor with a distinct preference for CDK9 (Ki values for CDK9/CycT is 3 nM *vs* 40–70 nM for other CDKs like cdk2). Unlike other cyclins which are involved in cell cycle,

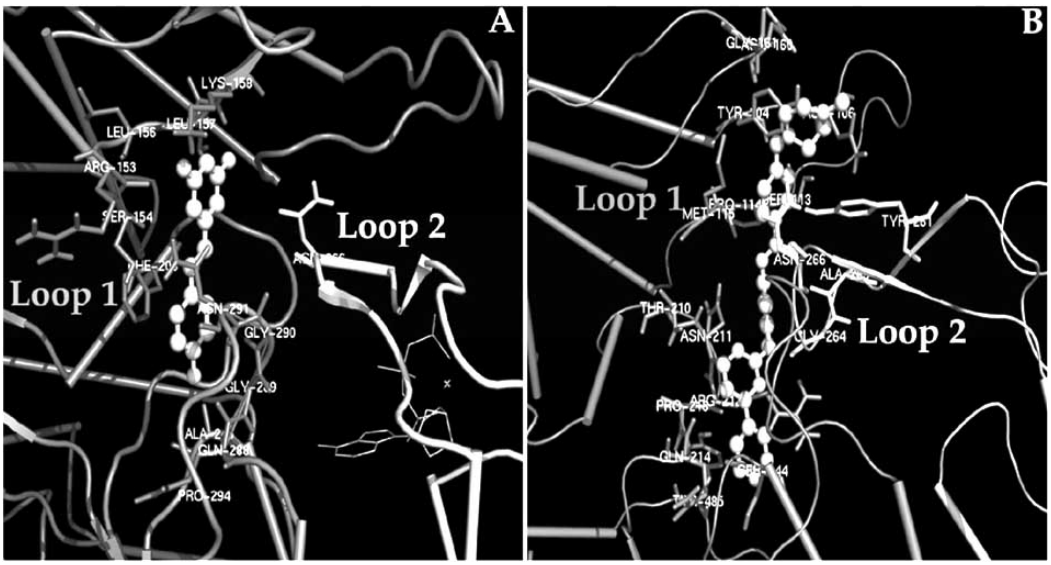


**Fig. (1).** Putative binding sites for resveratrol and gleevec on human ribonucleotide reductase. Stand alone version of autodock (4.2.1) was used to dock resveratrol (**A**) and gleevec (**B**) on human ribonucelotide reducatse. PDB 2WGH was used as the template and the bound ligands were removed prior to docking. Number of generation for each dock file was set to 75000. This figure is a representative of a high ranking cluster. 2WGH is in cartoon representation and dATP (white line) is superimposed in the model.

CDK9 is a transcriptional regulator [49]. Treatment of cells with flavopiridol results in a profound effect on transcription leading to decreased levels of a variety of mRNAs that belong to early transcription factors, cytokines, cell cycle regulators, kinases and antiapoptotic proteins such as Mcl-1 and XIAP [49]. Flavopiridol treatment therefore sensitizes cells to apoptosis [50]. These observations imply that CDK9 could be a relevant flavopiridol target.

In addition to its ability to directly bind CDKs, flavopiridol can also recognize other 'unrelated' proteins like glycogen phosphorylase (GP) [51, 52]. GP converts glycogen to glucose, and excess glucose inhibits break-down of glycogen leading to its storage. Flavopiridol treatment of A549 non-small cell lung carcinoma cells resulted in increased glycogen accumulation [51]. It is believed that by targeting GP, flavopiridol may have an added benefit in starving cancer cells of glycolytic intermediates. Glucose has a synergistic effect on flavopiridol administration and, at 10mM, lowers the concentration required for $IC_{50}$ to five fold [51].

Crystal structures of CDK9, glycogen phosphorylase and CDK2 in complex with flavopiridol have been solved to understand the molecular basis of the interaction [49, 51, 53]. Interestingly, in all three cases, the respective target seems to be locked in an inactive conformation by the inhibitor. In the CDK9/CycA complex, binding of flavopiridol renders CDK9 incapable of binding to ATP. The changes brought about by this binding are reportedly very similar to that seen with the Abl-gleevec complex. An analog of flavopiridol, deschloro flavopiridol binds to the inactive monomer of CDK2. In the case of GP, flavopiridol binds to the less active T-state. The mode of interaction and the functional groups involved (flavopiridol *vs*. deschloro flavopiridol) are different for the three different target proteins. Such diversity in interaction explains the all too often observed property of natural products - their ability to alter multiple pathways.

Gleevec and flavopiridol both bind to very different molecular targets, yet the mechanism of inhibition is very similar - the ability of the inhibitor (which binds non-covalently) to freeze the target protein in an inactive conformation. There may be other molecules which use similar strategy for inhibiting enzyme activity or protein function. It seems that drug screening methods may benefit by the knowledge about protein conformation, and it is likely that some of the molecular targets-especially those that undergo allosteric regulation, may be revealed by taking into consideration the role of protein conformation.

## 2. INHIBITORS OF PROTEIN-PROTEIN INTERACTION

### 2.1. Gossypol and its Analogs

Many polyphenols derived from plants have an anti-cancer effect. The most notable among those are present in green and black tea extracts. They inhibit cell proliferation and induce apoptosis in cancer cells [54, 55]. One of the mechanisms by which apoptotic cell death pathway is kept in check is through the hetero oligomerization of the pro-apoptotic (BH3 domain containing proteins) and anti-apoptotic proteins (Bcl-2 and Bcl-x) [54, 56]. Down regulation of the expression of Bcl-2 has been shown to sensitize cancer cells to chemotherapy. Bcl-2 anti sense oligonucleotide, a product from Genta has entered phase III clinical trial [57, 58] providing hope that small molecular mimics of BH3–Bcl-2 interactions could be potential anti-cancer agents. To identify the polyphenols from green and black tea extracts which would disrupt the BH3-Bcl-2 interaction, Nuclear Magnetic Resonance (BH3 peptide-Bcl-$x_L$interaction) and fluorescence polarization displacement assays (BH3 peptide-Bcl-$x_L$ interaction and BH3 peptide-Bcl-2) were used [56]. A peptide mimic of BH3 was used as a surrogate for the full length protein. By analyzing the structures of the compounds that displaced the BH3 peptides, the authors found that a gallate group as in (–)-epigallocatechin-3-gallate (EGCG), was important for the inhibitory effect of the polyphenols, and this structural requirement was common to both the green and black tea extracts. The assay system used in these studies is probably one of the most robust – the ability of the natural compounds to displace a bound ligand, and represents one of the most convincing evidence for the specificity of interaction with clear relevance in the *in vivo* context. Based on co-immunoprecipitation studies, another analog of gossypol was recently shown to prevent heterodimerization between Mcl-1/Bax and Bcl-2/Bim, again providing convincing evidence that the target of gossypol is the protein-protein interaction site [59].

This set of studies is also particularly noteworthy for the following reasons. Based on these mechanistic studies, the authors would go on to use the crystal structure of Bcl-2 to model the binding of these compounds. This led to the design of apogossypol, an analog with no free aldehyde groups. While the analog was less potent than the parent compound in inhibiting BH3 peptide-Bcl-2 interaction, it was as effective as gossypol in inducing cell death in tumor cell lines. More importantly, the analog was less toxic as it lacked reactive aldehyde groups thus preventing it from targeting other biomolecules inside the cells and compromising their activity. Removal of the functional group also resulted in maintaining the levels of the compound in solution, and prevented its loss through unwanted reactions. The increased stability probably also resulted in better uptake by the cells. This is a good reference for a design strategy – reactive groups are not always necessary especially when non-covalent interactions are involved. In a series of studies that followed, the group generated Bcl-2 transgenic mice and showed that oral administration of apogossypol was less toxic, reduced the size of the spleen and the number of B cell counts in spleen [19]. Other molecules that would bind EGCG at the physiologically relevant concentrations after dietary intake of tea (0.1-1 μM) would be 67 kDa laminin receptor (67LR Kd ~ 40 nM) [60], 20S proteasome (0.1-0.2 μM) [61], Bcl-2, Bcl-x (0.3 - 0.5 μM Ki) and vimentin (Kd 0.003 μM) [62].

### 2.2. Epidithiodiketopiperazine

Epidithiodiketopiperazine (ETP) family of compounds is credited with anti-cancer properties. Natural compounds containing this functional group like gliotoxin inhibit farnesyl and gerenaylgeranyltransferase while chaetoxin inhibits histone methyl-transferase. Although these compounds were well known for their anti-cancer activity, correlation between structure and activity was lacking. This was finally resolved by demonstrating that the CH1 domain of transcriptional co-

activator p300 is the direct molecular target of the disulfide core of an ETP family member chetomin. This binding results in the disruption of protein-protein interaction between hypoxia inducible factor HIF-α and p300 by a remarkable mechanism. ETP removes a well co-ordinated zinc atom from p300-CH1 by thiol-disulfide exchange [63]. Guided by protein structure, biochemical studies, mass spectrometric analysis of the reaction products and cell based assays to monitor the expression of a reporter gene of HIF-α, the authors provide evidence that chetomin acts by disrupting protein-protein interaction between HIF-α and p300. Some of the other ETP targets either bind zinc or are associated with zinc binding proteins. CH1 domain is shared by proteins that bind transcription factors. The authors also contemplate about the possibility that ETP disruption of zinc binding in such cases may account for the exceptional anti-tumor effect of ETP observed *in vivo*.

### 2.3. Inhibitors of MDM2-p53 Interaction

Ubiquitin Proteasome Pathway (UPS) is a well known molecular mechanism responsible for regulated degradation of various cellular proteins involved in functions ranging from transcription, DNA repair, antigen presentation, cell cycle, apoptosis and in the degradation of oncoproteins as well as tumor suppressor proteins [30, 64-66]. This pathway as a whole or the proteasome alone is also responsible for the destruction of truncated, misfolded and abnormal proteins. The proteins to be degraded are generally marked by a post-translational tagging mechanism wherein three enzymes E1, E2 and E3 by a series of reactions charge and load a small protein called ubiquitin to mark the protein to be degraded. Specificity in substrate recognition is primarily mediated through E3 also called the ubiquitin ligase [67]. Alteration in the levels of proteins related to tumor formation for example, stabilization of an oncoprotein and destabilization of a tumor suppressor are due to the aberrations observed in this pathway [67]. MDM2, an oncoprotein acts as an E3 ligase and ubiquitinates p53, a tumor suppressor, which is then destroyed by the proteasome [68]. Although MDM2 ubiquitinates other proteins inside the cells, the fact that p53 is one of the substrates for MDM2 had prompted investigators to look for inhibitors of this interaction. Structural studies provided the impetus with the identification of a small region of p53 bound to MDM2. Compounds that could prevent binding of the p53 peptide to MDM2 were screened and chalcones (1,3-diphenyl-2-propen-1-ones), hexylitaconic acid and chlorofusin were found to inhibit p53-MDM2 interaction [21]. This is a unique example where a tumor suppressor protein has been specifically targeted for a possible pharmacological intervention. However no dramatic progress has been made in the design of analogs with improved capabilities, partly due to the observed high toxicity of the compounds. p53 released from MDM2 was apparently unable to bind to DNA, and it was speculated that this compound may react covalently with p53. It may be recalled that elimination of reactive groups resulted in reduced toxicity of gossypol - an inhibitor of protein-protein interaction within the apoptotic family of proteins. Since the MDM2-p53 interaction is primarily non-covalent in nature, it may not be necessary to retain such reactive functional groups, removal of which may help prevent unwanted reactions and toxicity.

The MDM2-p53 interaction has nevertheless been successfully disrupted using synthetic small molecules which mimic the binding of p53 to MDM2 [69, 70]. Protein-protein interactions are not considered as attractive targets for drug design. Their interacting surfaces are relatively flat (non-enzymatic) and different from protein-ligand interactions [71]. With a well defined pocket, it is likely that there are other molecules in the natural product library that can interfere with the MDM2-p53 interaction.

## 3. COMPOUNDS WITH REACTIVE FUNCTIONAL GROUPS

### 3.1. Inhibitors of Proteasome

The proteasome - a self-compartmentalized ATP dependent protease, harbors three different types of active sites which are called chymotrypsin like (CT-L), trypsin (T-L), and post-glutamyl or caspase like (C-L). Each active site has unique sequence preferences, as seen from the small fluorogenic peptide substrates that they cleave and the nature of the peptide libraries used in positional scanning approaches. Several natural product based inhibitors of the proteasomes have been identified or synthesized. Lactacystin, - a natural product which contains the three units - leucine, isobutyrate (and/or valine), and cysteine, inhibits the activity of the proteasome and that of a lysosomal enzyme - cathepsin A. Lactacystin is however inert towards serine and other cysteine proteases [72]. The specificity of lactacystin for the proteasome and its lack of reactivity to other serine proteases have been explained on the basis of the chemistry of the active site. Lactacystin undergoes cyclization in solution to form a lactone, which is the reactive species. When the catalytic threonine in the proteasome was replaced by a serine, the reaction intermediate - an acyl-enzyme complex, was more rapidly hydrolyzed. This observation was extrapolated to argue that the serine proteases would rapidly destroy the lactone, while it would form a more stable complex with the proteasome. Likewise, MG132 - the synthetic derivative made up of three leucine residues and an aldehyde functional group, inhibits lysosomal enzymes in addition to the proteasomes [72]. Remarkably, a boronate functional group instead of the aldehyde (MG132), in an otherwise identical peptide scaffold, renders MG262 apparently more selective towards the proteasome. At higher concentrations however, cathepsin A is inhibited [73]. An elegant set of studies has demonstrated that in synthetic peptides containing a vinyl sulfone functional group, changing the amino acids at the distal site of binding can lead to exclusive labeling of a single active site of the proteasome [74].

There is a continuous effort to identify new inhibitors of the proteasome to overcome toxicity, drug resistance and differences in the effect of proteasome inhibitors on different cell types. This new class of inhibitors appears to be more effective than bortezomib [75]. Recently, NP1-0052, an orally active inhibitor, was identified from fermentation cultures of *Salinospora*, a new marine gram-positive actinomycete [73]. Ironically, this inhibitor turns out to be a close analog of omuralide - the active form of lactacystin A de-

scribed above. A combination of bortezomib and NPI-0052 at low doses was found to synergistically inhibit tumor growth in human plastocytoma xenograft, a mouse model for multiple myeloma (MM) [76]. The rationale to test the synergistic effect of two compounds which targeted the same molecule was based on the differences in structure and mechanism of action of the two compounds [73]. The synergy was also reflected in the inhibition of the three proteasomal (from tumor) active sites.

This example of achieving synergy in inhibition by targeting the same molecule must represent a unique case. This has been possible because the architecture of the proteasome is unique in that this self compartmentalized protease harbors three different active sites in its catalytic chamber. The specificity of each of the active sites is unique. By selectively inhibiting each individual active site on purified proteasomes, the authors clearly showed that the nature of the protein substrate dictated the relative roles of the active site [77]. However the *in vivo* role of the relative and independent role of three active sites have not been fully explored partly due to the lack of cell permeable inhibitors specific for each active site. It is evident from the experiments using the human plastocytoma xenograft mouse model for MM that NPI-0052 is more effective against the T-L activity as compared to bortezomib, and when both are present the inhibition is further enhanced. How this synergistic inhibition reflects or explains the different mechanism of action of the two compounds remains to be characterized. However, NPI-0052 was found to have a pronounced effect on FADD–caspase-8–mediated cell death signaling in MM distinct from the effect of bortezomib [73]. Whether elimination of some of the substrates of this pathway is dependent on the T-L active site of the proteasome would be an attractive hypothesis to test.

The proteasome inhibitors in clinical use are unique in that they are peptide based or are peptide mimetic. Bortezomib is a synthetic inhibitor carefully designed based on the mechanism of action of the proteasome, active site chemistry and substrate specificity. Nature seems to have an antidote for the proteasome activity in lactacystin, for example, complete with the mechanistic details.

### 3.3. Fumagillin

Methionineaminopeptidases (MetAp) are enzymes that selectively catalyze removal of initiator methionine residue from a polypeptide chain. However the enzyme from yeast, rat and human does not seem to have any amino peptidase activity [78]. Fumagillin and ovalicin are structurally related natural products that potently inhibit angiogenesis by blocking endothelial cell proliferation. Fumagillin binds irreversibly to the His-231 of methionine aminopeptidase 2 through its reactive ring epoxy group and inhibits the enzyme activity [79]. Specificity of this binding is reiterated by the observation that a closely related type 1 enzyme, - MetAP1, is not inhibited. This was correlated to the presence of an inactivating Thr instead of Ala at position 362 in MetAP1 [80]. In this example, a protein specific scaffold in the ligand dictates target specificity even when a highly reactive functional group is present. The same principle is reflected in the exclusive labeling of human carbonic anhydrase II with affinity probes [81]. In this instance, the enzyme was deliberately spiked into yeast cell proteome and ligand containing the epoxy group labeled the enzyme specifically. Such probes are constantly used in proteomic studies and would be very useful in the design of potent natural product derivatives.

### 3.4. Pladienolide

Pladienolide B from a *Streptomyces* culture was found to be a potent inhibitor of hypoxia induced - VEGF expression and proliferation in U251 cancer cell line [82]

The molecular target was long sought after and cleverly designed molecular probes like $^{3}$H-labeled, fluorescence - tagged and photoaffinity/biotin (PB)-tagged pladienolide finally led to the identification of a splicing factor as the key target of pladienolide [83]. This rationalized the anti - tumor effect of pladienolide observed *in vitro* and *in vivo*. In human lung cancer LC-6-JCK xenograft model, E7107 - one of the derivatives, apparently caused complete tumor remission with a wide therapeutic window.

The field of drug discovery aimed at target specific inhibition used to be dominated by inhibitors either designed or screened for the ATP or nucleotide binding pockets, many of which are kinases. Undoubtedly they contribute to a large number of effective compounds with a great promise in clinical use. Examples listed in this review indicate that we also have a variety in the choice of functional groups and scaffolds at our disposal (Table **1**). The molecular targets span a wide spectrum of proteins like apoptotic proteins, proteases, tumor suppressor proteins, transcription factors, enzymes involved in splicing, etc. Many advances have contributed to shifts and expansion in this field, and they continue to do so. High throughput mass spectrometric identification of the targets [84] and computational methods [85, 86] have accelerated the process of identification of novel molecular targets. There is an increase in the knowledge gained from structure activity relationships. As an illustration, we have provided results from our on-going efforts to characterize putative molecular targets of some of the natural products like gleevec and resveratrol using computational methods.

In summary, the case studies discussed here emphasize subtlety inherent to molecular interactions and in the reaction mechanisms mediated by natural products. The examples also highlight how difficult it is to successfully translate the delicate balance between target and off target interactions into pharmacologically relevant target selectivity *in vivo*. Exceptional selectivity may actually be detrimental as it tends to generate drug resistance. Diligent efforts, careful structure activity studies, and the acceptance that natural products will bind to multiple targets and such interactions in a majority of the cases is likely to be specific, will help us harness the immense potential of natural resources in anticancer drug discovery program. Combining two or more drugs that can bind to multiple targets from different pathways is a viable strategy which has been tested in a limited number of cases. It is also possible to achieve a synergistic effect by molecular tinkering of a single natural product which binds to two or more targets from different pathways. With such choices available, treating advanced stages of

**Table 1. Molecular Targets of Natural Products and other Compounds**

| Compound | Target | | References |
|---|---|---|---|
| *Changes in protein conformation* | | | |
| Gleevac (Imatinib) | BCR-ABL<br>Other targets –PDGFR, c-Kit, NQO2 | CML | [33, 87]<br>[33] |
| Resveratol | NQO2, Ribonucleotide reductase.<br>Other targets F0F1 ATPase, CDK7, DNA polymerase 1 | Mouse mammary gland culture model of carcinogenesis and in two-stage mouse skin cancer model. | [27, 37-42] |
| Flavopiridol | CDK9 Glyocogen phosphorylase, CDK2 | Flavopiridol is currently in clinical trials as a CDK9 inhibitor for a number of cancers, with promising results for the treatment of chronic lymphocytic leukemia (CLL) | [48-52, 88] |
| *Inhibition of Protein-protein interaction* | | | |
| Gossypol and apogossypol | Bcl-2/Bim, Laminin receptor, 20S proteasome, Vimentin | Gossypol and apogossypol display cytotoxic activity against<br>cultured human B-cell lymphoma and leukemia cells; apogossypol displayed superior activity to gossypol in terms of reducing splenomegaly and reducing<br>B-cell counts in spleens of Bcl-2– transgenic mice. The (_) enantiomer of gossypol (AT-101; Ascenta Pharmaceuticals, San Diego, CA) is currently under<br>clinical evaluation in phase 1/2 clinical trials involving patients<br>with solid tumors, lymphoma, and leukemia. | [56, 59-61] |
| Epidithiodiketopiperazine compounds - chaetoxin | Histone methyl transferase<br>P300 | Effective against Xenografts generated with HepG2 EPO-Luc cells in NCr/Nude mice | [63] |
| Chalcones, Hexylitaconic acid,chlorofusin;<br>Nutlin the small molecule inhibitor | p53-MDM2 interaction | Toxic against normal cells;<br>Nutlin however suppressed the growth of established tumor xenografts of human osteosarcoma cell line SJSA-1 | [21, 70]<br>[70] |
| *Compounds with reactive functional groups* | | | |
| Lactacystin, NPI-0052;<br>Bortezomib the synthetic peptide mimic | Proteasome | Bortezomib effective against Multiple Myeloma (MM); NPI-0052 and bortezomb induce synergistic anti-MM activity both *in vitro* using MM cell lines or patient CD138(+) MM cells and *in vivo* in a human plasmacytoma xenograft mouse model | [22, 73, 89] |
| Fumagillin | MetAP 2 | Fumagillin derivative was in clinical trials for solid tumor<br>and Kaposi's sarcoma. Compound showed neurotoxicity | [78, 79, 90] |
| Pladienolides | Splicing factor | In the human lung cancer LC-6-JCK xenograft<br>model, E7107 - a pladienolide derivative, caused complete tumor remission with a wide therapeutic window. It is in clinical trial. | [82] |

cancer and providing a solution for the long term management of the disease may eventually become a reality.

## ABBREVIATIONS

CML = Chronic Myelogenous Leukemia

MM = Multiple myeloma

MetAp = Methionineaminopeptidases

ATP = Quinone reductase NQO, Adenosine triphosphate

NADH = Nicotinamide adenine dinucleotide

NADPH = Nicotinamide adenine dinucleotide phosphate

CDK = Cyclin Dependent Kinase

RNR = Ribonucleotide reductase

GP = Glycogen phosphorylase

ETP = Epidithiodiketopiperazine

CT-L = Hypoxia inducible factor HIF, Chymotrypsin like

T-L = Trypsin like

C-L = and Post-glutamyl or Caspase like

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