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Structural Analysis of Flavonoid/Drug Target Complexes: Natural Products as Lead Compounds for Drug Development

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Abstract

Plants produce pigments called flavonoids that are synthesized (de novo) primarily from the amino acid phenylalanine. Many natural products such as herb preparations for medicinal use contain flavonoids and so these diverse structural compounds have become potential lead compounds for a variety of illnesses ranging from specific cancers to gout. Recently, Biotechnology has been utilized through bioorganic engineering to manufacture flavonoid and modified flavonoids for medicinal applications. A growing number of protein-flavonoid complexes have been crystallized and their structures solved in the last decade. A summary of the protein-flavonoid complexes with medicinal relevance is presented herein. A detailed analysis of selected protein-flavonoid complexes is provided. The goal is to provide insights for modifications to known flavonoids that could be biomanufactured to generate more specific and efficient binding of flavonoids to protein targets with medicinal relevance.

Keywords: Protein-ligand complexes; Flavonoids; Drug development; Biomanufacturing; Structural biology; Molecular docking

The Relevance of Flavonoids for Medicinal Chemistry and Drug Development

Flavonoids are plant pigments that are synthesized (de novo) from phenylalanine. In medical preparations with herbal and insect components, flavonoids play an important functional role [1]. Recently, flavonoids have been investigated as new approaches to drug therapy that targets different kinds of illnesses [1]. Natural flavonoids that have been purified, inhibit specific enzymes to stimulate some hormones and neurotransmitters [1]. There are seven classes of natural flavonoids, as shown in Figure 1: Flavanones, flavones, flavonols, isoflavone, chalcones, aurones, and proanthocyanidins [2]. There are examples in the literature from all seven classes of flavonoids that illustrate their potential use for disease treatment [1,2]. Many types of enzymes are inhibited by several classes of the flavonoids, including: hydrolases, oxidoreductases, DNA synthases, RNA polymerases, phosphatases, protein phosphokinases, oxygenases, and amino acid oxidases. The inhibition may be competitive but more often it is allosteric inhibition [1] which may cause architectural changes in the active site of the target enzyme. Although flavonoids affect almost all enzyme classes, they do not cause damage in metabolism due to low solubility in water so concentrations are relatively low and these biologically active molecules have short half-life *in vivo* [1].

Flavonoids are known as secondary plant phenolic compounds which are widely distributed with different metabolic functions in plants [1,2]. The biosynthetic pathway of flavonoids is known, as well as the regulation by various transcription factors. In recent years, biotechnology has been used through metabolic engineering to generate flavonoid products for medicinal applications [2]. In addition, strategies of genetic engineering of flavonoid biosynthesis for industry and the combinatorial synthesis in microorganisms are being performed to biomanufacture (reconstruct the pathway of flavonoid synthesis) with high yield of specific flavonoids for medicinal purposes [2].

Many flavonoids have cardio-protective effects that are derived from the ability of these molecules to inhibit lipid peroxidation [3]. Flavonoids occur in foods primarily as glycosides and polymers that are degraded in the digestive tract [3]. Flavonoids are a class of constituents of phenolic compounds which are found in fruits and vegetables. Fruits and vegetables are rich in compounds (such as flavonoids) with phenolic constituents and beta-carotene. Flavonoids such as (-)-epicatechin is present in dark chocolate and is responsible for its antioxidant properties [4]. The flavonoids have an antioxidant capacity and may be a significant contributor to the lowered disease risk as antimicrobial agents [4,5]. Flavonoids are present in soy, vegetables, fruits, cereals, nuts, and beverages (red wine, beer, tea, and cocoa) [6].

Flavonoids have an anti-microbial function such as direct antibacterial activity, which works synergistically with antibiotics, to combat bacterial infections [5]. In drug discovery, new interest has developed to utilize flavonoids as lead compounds to create new anti-bacterial drugs with better resistance. The antimicrobial activity of flavonoids, a class of natural products that possess a diverse range of pharmaceutical properties [7] is summarized and reviewed herein along with selected structural comparisons of known protein/flavonoid complexes in the Protein Data Bank (PDB).

Current natural product research has found that flavonoid-rich plant foods or food extracts have significant improvement in protecting memory and learning. Flavonoids stimulate neuronal regeneration. These compounds are beneficial as well in related dementias and Alzheimer Disease (AD). Furthermore, flavonoids may also serve as protective agent in the pathology of Parkinson 's Disease (PD) [8].

In addition, flavonoids are being actively pursued as anti-cancer agents. More specifically, "the fourth most common malignancy and second leading cause of death due to cancer worldwide is gastric cancer" [9]. In a recent research study a modest decreased risk of gastric and lung cancer was confirmed by diet rich in flavonoids [10]. The study reported that eating fruits and vegetables, which are rich in flavonoids, most likely protect people against cancers of the mouth, pharynx, larynx, esophagus, and stomach. These cancers that may be deterred by natural products, are related to smoking which classify the cancers as a cause of cancer of the lungs, oral, cavity, nasal and

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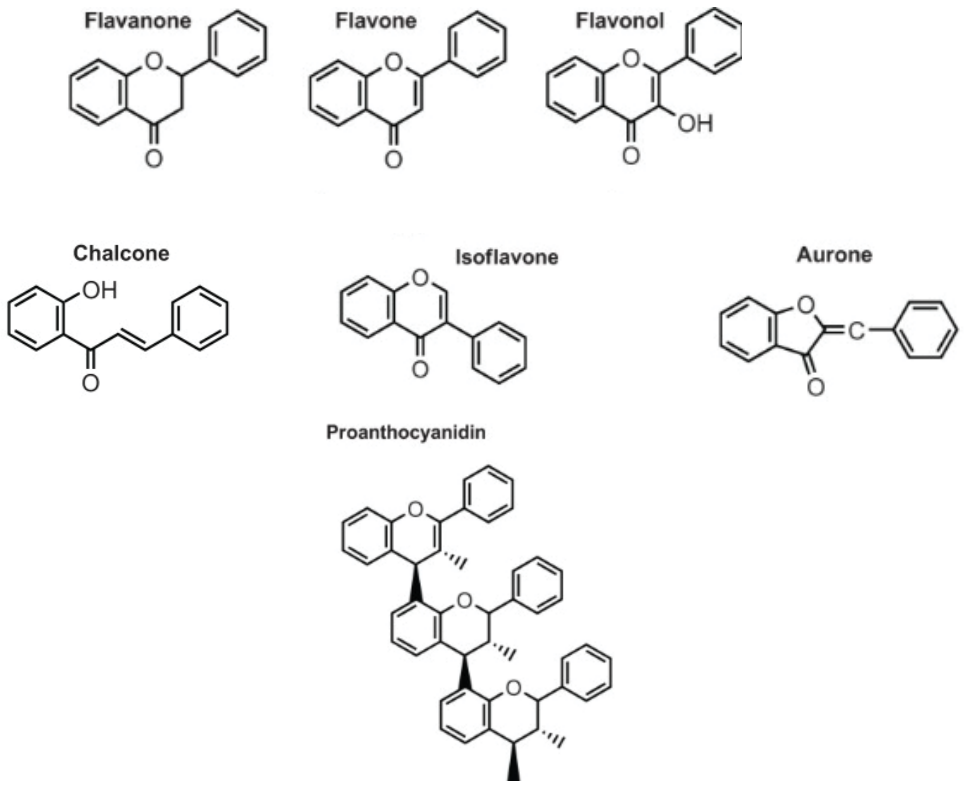

Figure 1: Representative Flavonoids Commonly Found in Human Diet.

paranasal sinuses, pharynx, larynx, esophagus, kidney, liver, uterine cervix, stomach, bladder, pancreas, as well as myeloid leukemia [10].

The fact that flavonoids exhibit structurally diverse patterns; has resulted in these molecules being recognized as a rich source of compounds with potential anti-cancer properties. The flavonoids block the cell cycle causing apoptosis and disrupts mitotic spindle formation, which is needed for cell division. Therefore, flavonoids make them promising not only for therapeutic drugs for many other illnesses but also make them promising lead compounds in anti-cancer research [11].

A Structural Library of Flavonoid/Protein Complexes

Selection of flavonoid/protein complexes

A library of selected flavonoid/protein complexes was built from searches of the Protein Data Bank (PDB) for both human and non-human structural entries. A total of 47 entries were found for human structural flavonoid/protein complexes and another ~ 65 structural hits for non-human complexes including molecules that synergistically interact with flavonoids to render them effective putative cancer treatment/prevention compounds for specific cancers such as prostate cancer [12]. The complete library is provided as supplemental materials to this review in Appendix A: Flavonoid/Protein Complexes. The Appendix lists the structures of the flavonoids and the ligands with IUPAC nomenclature as well as the associated PDB codes. Selected flavonoid/protein complexes that were analyzed in detail are shown in Table 1. In addition to relevance for lead compounds for drug development, the structural criteria for selection for both the human and non-human flavonoid/protein complexes of PDB x-ray structures for detailed analysis was: 1) Resolution of 2.90 angstroms or lower; 2) A redundancy of 3.5 or higher overall and 1.8 angstroms or higher in the highest resolution shell; 3) a signal to noise ratio of 1.5 or higher in the highest reported resolution shell; and R_{factor} of 0.24 or lower and R_{free} less than 0.28. Table 1 summarizes the PDB's of flavonoid/protein complexes (with the highest resolution) that are discussed in this review. These flavonoids are co-crystallized with protein targets.

Overview of Flavonoid/Protein Complexes Relevant for Drug Development

Part I Structural flavonoid/protein complexes of medicinal significance

Targeting diabetes: The structure of human pancreatic α-amylase in complex with inhibitory flavonols and ethyl caffeates has yielded information about how these molecules may inhibit the enzyme (PDB code 4GQR) [13]. α-Amylase cleaves starch to liberate glucose so inhibition of this enzyme potentially drops glucose levels in the blood. The inhibition of glycogen phosphorylase makes this enzyme a target for drugs for Type II Diabetes (PDB code 3EBP) [14]. Flavonoids have been discovered to inhibit glycogen phosphorylase (GP), a target to control hyperglycemia in type 2 diabetes. The crystal structure of the GPb-chrysin complex was determined in order to understand how the inhibition works. "Chrysin is bound at the inhibitor site sandwiched between the aromatic side chains of Phe285 and Tyr613 through hydrophobic interactions. Chrysin binds to GPb approximately 15 times weaker (Ki=19.01 μM) than flavopiridol (Ki=1.24 μM), exclusively at the inhibitor site, and both inhibitors display similar behavior with respect to AMP. To identify the source of flavopiridols' stronger affinity, molecular docking with Glide and post docking binding free energy calculations using QM/MM-PBSA were performed and compared" [14]. Although docking failed to correctly rank inhibitor binding conformations, the QM/MM-PBSA method was superior to model the hydrophobic (stacking) interactions correctly and reproduce

PDB Code	Organisms	Protein	Flavonoid bound to protein/ID from Protein Data Bank	References
4GQR	*Homo sapiens*	Pancreatic alpha-amylase	Myricetin (MYC)	[13]
3EBP	*Oryctolagus cuniculus*	Glycogen phosphorylase (GPb)	Chrysin (weak binding not listed in PDB) Flavopiridol (CPB)	[14]
2H44	*Homo sapiens*	cGMP-specific 3',5'- cyclic phosphodiesteras (PDE5A1)	Icarisid II (7CA)	[17]
2H42	*Homo sapiens*	cGMP-specific 3',5'-cyclic phosphodiesteras (PDE5)	Sildenafil,Viagra (VIA)	[17]
3NG5	*Homo sapiens*	V30M Transthyretin (TTR)	Epigallocatechin gallate (EGCG) (KDH)	[18]
4DES	*Homo sapiens*	Transthyretin(TTR)	Chrysin (57D)	[19]
4DEW	*Homo sapiens*	Transthyretin (TTR)	Luteolin (LU2)	[19]
4DET	*Homo sapiens*	Transthyretin (TTR)	Kaempherol (KMP)	[19]
4H2B	*Homo sapiens*	Ecto-5'-nucleotidase	Baicalin (OXE)	[20]
1C8K	*Oryctolagus cuniculus*	Glycogen Phosphorylase (GP)	Flavopiridol (CPB)	[23]
1E1Y	*Oryctolagus cuniculus*	Glycerol-3-Phosphate Dehydrogenase	Flavopiridol (CPB)	[23]
2JJ2	*Bos taurus*	ATP Synthase Subunit Alpha Heart Isoform (F1-ATPASE)	Phytopolyphenol, Resveratrol & Piceatannol (weak binding not listed in PDB) Quercetin *(QUE)*	[24]
3NVY	*Bos taurus*	Xanthine dehydrogenase/oxidase	Flavin-Adenine Dinucleotide (FAD) Quercetin (QUE) MTE	[25]
1FP2	*Medicago sativa*	Isoflavone O- Methyltransferase (IOMT)	Isoformononetin (HMO)	[26]
1CGK	*Medicago sativa*	Chalcone Synthase (CHS)	Naringenin (NAR)	[27]
1FM7	*Medicago sativa*	Chalcone-Flavonone Isomerase 1 (CHI)	7-hydroxyflavanone (weak binding not listed in PDB) 5-Deoxyflavanone (DFV)	[28]
1JEP	*Medicago sativa*	Chalcone-Flavonone Isomerase 1	4'-Hydroxyflavanone (DFL)	[28]
3BXX	*Vitis vinifera*	Dihydroflavonol 4-reductase (DFR)	Quercetin (QUE)	[29]
3C1T	*Vitis vinifera*	Dihydroflavonol 4-reductase (DFR)	Myricetin (MYC)	[29]
2C29	*Vitis vinifera*	Dihydroflavonol 4-reductase (DFR)	(2R,3R)-Trans-Dihydroquercetin (DQH)	[30]
2C1Z	*Vitis vinifera*	UDP-Glucose Flavonoid 3-O Glycosyl Transferase (VvGT1)	Kaempherol (KMP)	[31]
2C9Z	*Vitis vinifera*	UDP-Glucose Flavonoid 3-O Glycosyl Transferase (VvGT1)	Quercetin (QUE)	[31]
3HBF	*Medicago truncatula*	Flavonoid 3-O-glucosyltransferase	Myricetin (MYC)	[32]

Table 1: A Summary of Protein/Flavonoid Complexes.

experimental results [14]. Favopiridols' greater binding affinity may be a result of favorable interactions of the cationic 4-hydroxypiperidin-1-yl substituent with GPb, with desolvation effects limited by the substituent conformation adopted in the crystallographic complex [14]. Successful predictions were repeated using QM/MM-PBSA for the flavonoid quercetagetin (which binds at the allosteric site), and the data strongly suggests this computational methodology as an economic and accurate tool to predict flavonoid binding [14]. As shown in Figure 2, the human pancreatic alpha-amylase in complex with myricetin (MYC) PDB code 4GQR shows a hydrophobic pocket flanked by try59 and Tyr62 (Figure 2). Adjacent to the hydrophobic binding pocket (upper left of Figure 2) is a cluster of hydrophilic residues that have shown to be like a pocket in CASTp studies [15] and may be a novel allosteric binding site for modified flavonoids as moderate scores in DOCK6 [16] were obtained when testing Myricetin in "blind" docking studies (Averaged coordinates of the hydrophobic binding pocket--data not shown). Alternatively binding of MYC derivatives could be more specific and potent for the pancreatic alpha-amylase by enhancing hydrogen bonding by adding hydrophilic functional groups to increase potential hydrogen bonding adjacent to the metal binding site at His201, His101, Asp197, and Thr163.

Pulmonary hypertension and erectile dysfunction (PDB codes 2H44 and 2H42): Phosphodiesterase-5 (PDE5) is the target for sildenafil, vardenafil, and tadalafil, which are drugs for treatment of erectile dysfunction and pulmonary hypertension [17]. The crystal structures of a fully active catalytic domain of apoenzyme PDE5A1 and its complexes with sildenafil or icarisid II have been solved and deposited PDB codes 2H44 and 2H42 [17]. PDE5 belongs to a large family of cyclic nucleotide PDEs that catalyze cAMP and cGMP hydrolysis. "These structures together with the PDE5-isobutyl-1-methylxanthine complex show that the H-loop (residues 660-683) at the active site of PDE5 (A1 isozyme) has four different conformations and migrates 7-35A upon inhibitor binding" [17]. Phosphodiesterase-5 (PDE5) is shown in complex with Icarisid II (7CA) in PDB code 2H44 in Figure 3. Analysis of the details of the intermolecular interactions reveals that, by adding hydrophobic moieties such as isopropyl groups to 7CA, a change may be achieved which would enhance hydrophobic interactions to the right of Tyr664 near the interior of the hydrophobic pocket (Figure 3). In addition, hydrophilic moieties (such as alcohol groups) on modified 7CA could enhance binding near the zinc and magnesium binding sites.

Preventing neurodegenerative diseases (PDB code 3NG5): "Amyloid fibril formation is associated with protein misfolding

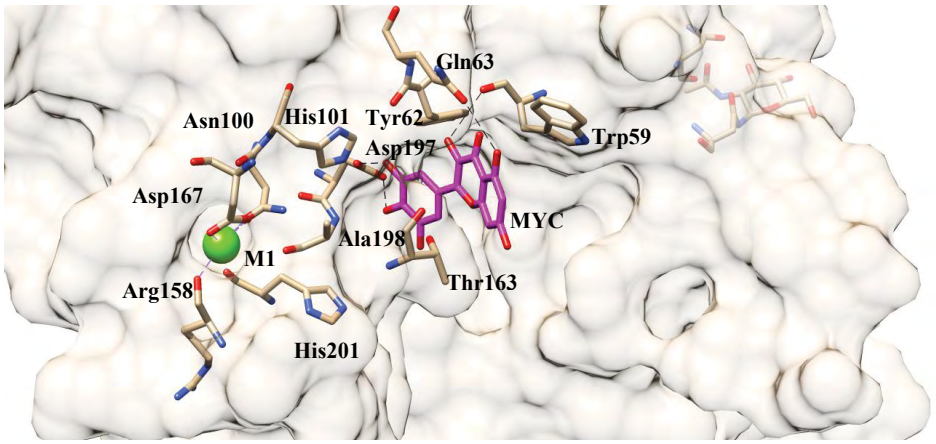

Figure 2: Diabetes Target: The Human Pancreatic α-amylase in Complex with Mericetin (MYC).

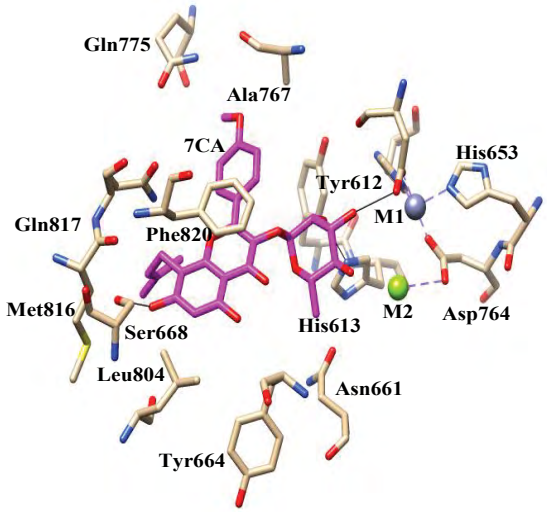

Figure 3: Pulmonary Hypertension Target: Phosphodiesterase-5 (PDE5) in Complex with Icarisid II (7CA).

disorders, including neurodegenerative diseases such as Alzheimer's, Parkinson's, and Huntington's diseases (18) Familial Amyloid Polyneuropathy (FAP) is a hereditary disease caused by a point mutation of the human plasma protein, transthyretin (TTR), which binds and transports thyroxine (T4)"[18]. TTR variants contribute to the pathogenesis of amyloidosis by forming amyloid fibrils in the extracellular environment [18]. Recently, researchers discovered that epigallocatechin 3-gallate (EGCG), the major polyphenol component of green tea, binds to TTR and suppresses TTR amyloid fibril formation. A novel binding sites distinct from the thyroxine binding site was discovered. V30m transthyretin, complexed with epigallocatechin gallate (EGCG) (PDB code 3NG5), is shown in Figure 4. Docking studies again revealed that EGCG may also bind at Thr119, Ser117, Asp18 and Val20 adjacent to one of the binding sites for EGCG. Perhaps a modified EGCG with enhanced hydrophilicity would increase affinity at current sites shown in complex and provide increased binding at the novel site enhancing the effects of the flavonol on transthyretin.

In addition, other flavonols may work synergistically with EGCG to enhance the reported effects flavonols have on this protein. These observations strongly suggest the possibility that EGCG may be a candidate compound for selected neurodegenerative diseases [18].

TTRwt binding chrysin (ttrwt_chr), ttrwt binding luteolin (ttrwt_lut), and ttrwt binding kaempferol (TTRwt_KAE):

Preventing neurodegenerative diseases: Transthyretin (TTR), a carrier protein in blood serum and cerebrospinal fluid for thyroid hormone Thyroxin (T4) and retinol, is considered a current target to treat Alzheimer's disease [19]. TTR is known to be involved in human amyloidosis. Small molecules are being developed that may act as TTR amyloid inhibitors. To develop new drugs, researchers co-crystallized the wild-type TTR and amyloidogenic mutant TTR V30M with several flavonoids (PDB codes 4DES, 4DEW, and 4DET [19]. In addition, assays were utilized to evaluate TTR aggregation in the presence of different flavonoids. Once effective flavonoid aggregation inhibitors were identified these flavonoids were analyzed by isothermal titration calorimetry (ITC) for binding affinity to TTRwt and mutant V30M. The tightest binding flavonoids were then utilized in the co-crystallization process. Detailed analysis of the structures reveals that changing the number and the position of the hydroxyl groups attached

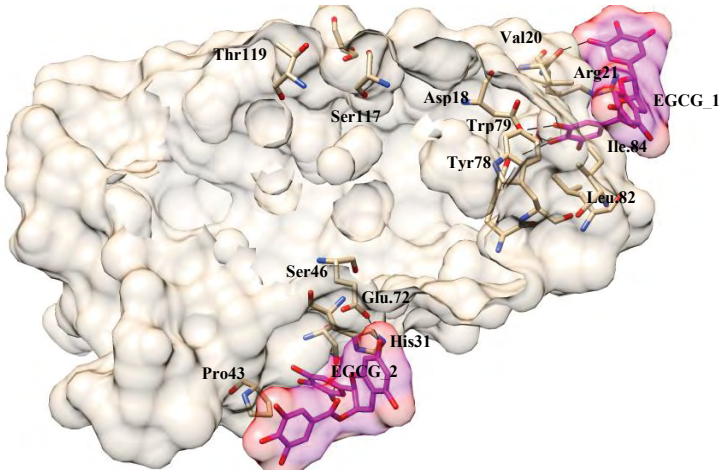

Figure 4: Neurodegenerative Disease Target: V30M Transthyretin Complexed with Epigallocatechin Gallate (EGCG).

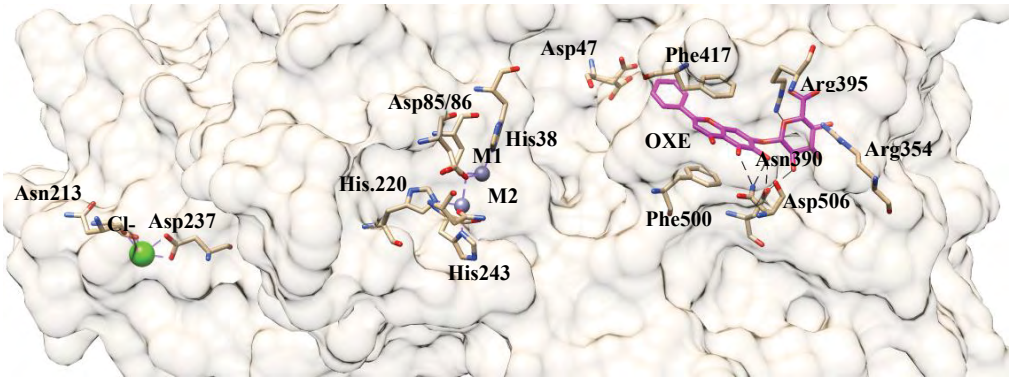

Figure 5: Inflammation, Hypoxia and Cancer Target: Ecto-5-nucleotidase (CD73) Complexed with Baicalin.

to the flavonoid core does effect binding of the flavonoid derivative to TTRwt and V30M. When the TTRwt and V30M apo form mutant were analyzed structurally, it was determined that the TTRwt binding site is highly selective and small changes in ligands tested had significant effects on binding in the central cavity of the binding site. In addition, the Met30 amyloidogenic mutation changes the architecture of the central cavity and the binding affinity to the flavonoid derivatives. This study illustrates that the mutant protein ligand binding specificity is altered from TTRwt. The mechanism of binding for ligands to the wild-type and the mutant TTR are explained and flavonoid binding interactions with the proteins are evaluated. Since such mutations exist (from clinical studies) that are known to correlate with increased amyloid production, this study is highly relevant for drug development [19].

Treatment of inflammation, chronic pain, hypoxia, and cancer (PDB code 4H2B): Ecto-5'-nucleotidase (e5NT) is considered an important drug target for the treatment of inflammation, chronic pain, hypoxia and cancer [20]. This is due to the fact that e5NT plays a critical role to activate adenosine receptors (P1) [20]. The hydrolysis of extracellular AMP to yield adenosine and inorganic phosphate is catalyzed by e5NT in vertebrates. This enzyme represents a crucial control point for the amount of extracellular adenosine. The crystal structure of the human e5NT has been determined and shows a dimer that undergoes large conformational changes between the active and inactive forms of the enzyme. In the activated state, the interface of the dimers assembles with the C-terminal domains, exhibiting approximately 13-degree change from inactive to active forms of the enzyme. A comparison of the inactive and active forms of the enzyme with and without AMP_CP shows that the control of the domain movement determines selectivity for monophosphate nucleotides [20]. In the open form of e5NT, when flavonoid derived compounds and nucleotide analogues are bound to the C-terminal domains, an additional binding pocket of 210 angstroms is exposed. This novel pocket is currently being investigated in order to design better inhibitors [20].

Interestingly, small molecules that may block metal cofactor binding of the Ecto-5-nucleotidase (CD73), complexed with baicalin (OXE) in PDB code 4H2B, may enhance binding of OXE at adjacent binding site in a negative allosteric effect (Figure 5). Once again, derivatives of flavonols with hydrophilic moieties or in this case secondary amine derivatives may bind tightly to enhance effects as tested in docking studies to be reported in full in future work.

In Figure 6, glycogen phosphorylase complexed with flavopiridol (CPB) is shown (PDB code 1C8K). By extending the CPB with ethylene groups, then hydrophilic functional groups towards the PLP binding pocket, binding scores increased 1000-fold with AutoDock [21] and confirmed in Glide with QM/MM-PBSA free energy calculations [22]. Enhanced binding to Tyr613, Glu572, and Lys574 was seen in these docking studies with CPB "derivatives".

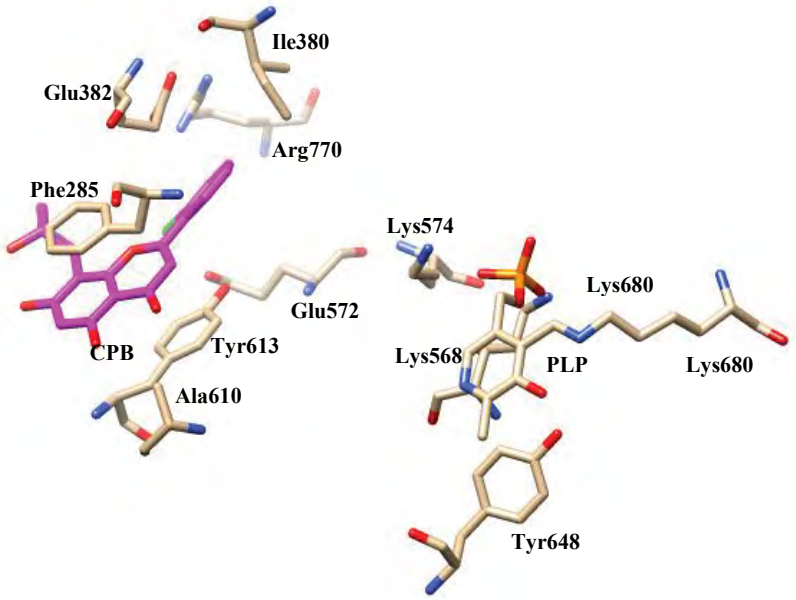

Figure 6: Targeting Tumor Cells: Glycogen Phosphorylase Complexed with Flavopiridol (CPB).

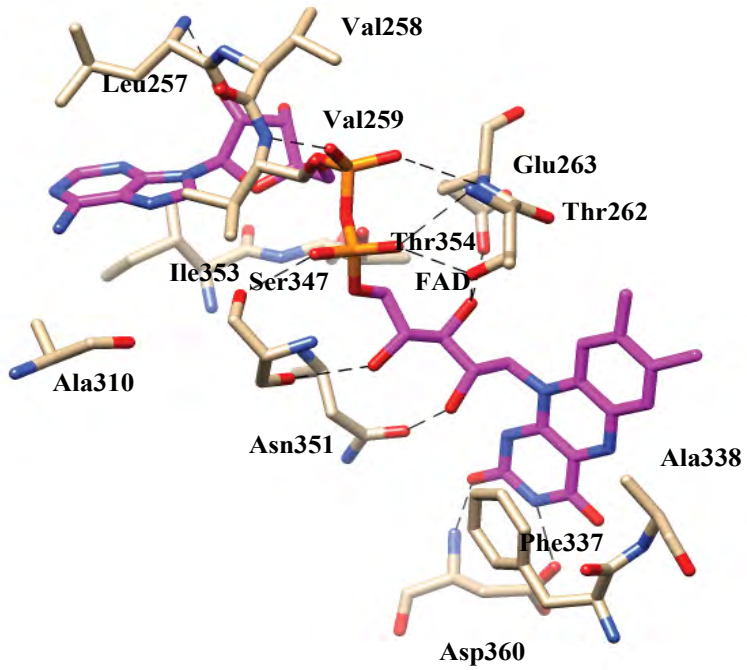

Figure 7A: Inhibition of Xanthine Oxidase: Bovine Xanthin Oxidase Complexed with Flavin-Adenine Dinucleotide (FAD).

Potent anti-tumor drugs currently in phase II trails PDB codes 1C8K and 1E1Y: Flavopiridol (L86-8275) ((-)-cis-5,7-dihydroxy-2-(2-chlorophenyl)-8-[4-(3-hydroxy-1-methyl)-piperidinyl]-4H-benzopyran-4-one), currently in phase II trials, is thought to be a potent antitumor drug that inhibits muscle glycogen phosphorylase (GP) [23]. The binding of L86-8275 to GP causes glycogen accumulation in non-small cell lung carcinoma cells (A549) [23]. GPb is inhibited by flavopiridol in the low micromolar range. The inhibition is enhanced with glucose decreasing the IC50 ~ 5-fold. This inhibition is very similar to the inhibitory effects of caffeine [23]. The structures of GPa , complexed with both glucose and flavopiridol GPb complexed with flavopiridol, and GPb, with caffeine have been determined and analyzed, to understand binding modes of flavopiridol to GP. Flavopiridol binds to the same site as caffeine, the allosteric inhibitor site next to the opening of the catalytic site. Phe285 and Tyr613 aromatic rings of GPb interact with flavopiridol, which is "sandwiched, between these two residues. The 280's loop of GPa, complexed with flavopiridol and glucose, is in the closed T position, which is preventing molecule interaction in the

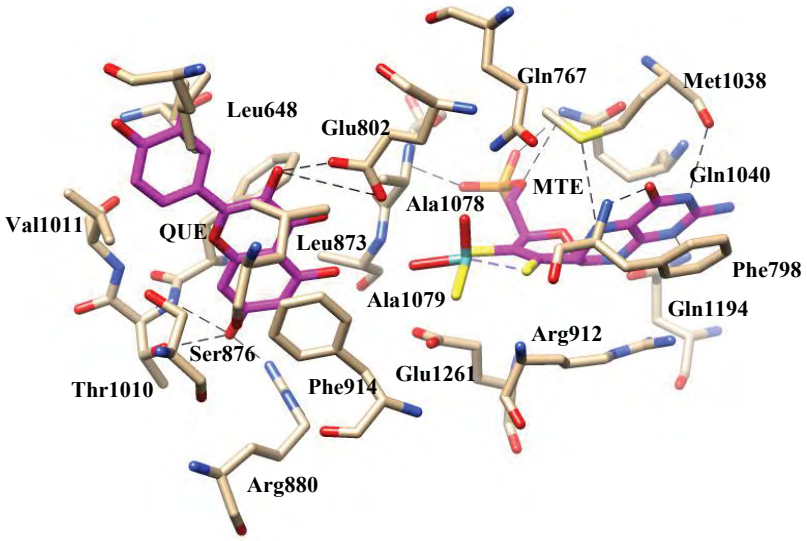

Figure 7B: Xanthine Oxidase Target: A Modified Molecule Combining QUE and MTE Docked into the Xanthine Oxidase Active Site.

catalytic site. The structural analysis explains the synergistic inhibition of glucose and flavopiridol. Structural analysis showed that different moieties of the inhibitor provided specific and potent binding to the GPa and GPb enzymes.

Targeting tumor cells: resveratrol mode of action (pdb code 2jj2): In order to investigate Resveratrol's mode of action, the bovine heart mitochondrial F(1)-ATPase structure has been determined in complex with dietary inhibitors phyto polyphenol, and resveratrol. The structures of the F(1)-ATPase, with the related polyphenols quercetin and piceatannol, have also been determined. The common binding site for the inhibitors is the inner surface of a cavity in the beta strands of the structure's N-terminal domains. The binding pocket is formed from loops in the three alpha and three beta subunits. Interestingly, by the N-terminal region of the alpha and beta subunits an interface occurs that enables the gamma subunit inside the cavity to rotate during catalysis [24]. More specifically, the F (1)-ATPase inhibitor binding site of the N-terminus of the alpha and beta strands is a hydrophobic pocket that interacts with the C-terminal end of the gamma subunit. The beta subunit interacts directly with the gamma subunit via hydrogen bonds to hydroxyl groups on the inhibitors. Water molecules participate in the binding and hydrophobic interactions of the subunit residues hold the binding pocket together. Only the beta "TP" subunit interacts directly with the gamma subunit. Beta "DP" and Beta "E" do not bind to the gamma subunit. The proposed mode of action of the inhibitors is that all types of rotation of the gamma subunits are blocked by inhibitors. Dietary resveratrol and related compounds may induce apoptosis because these inhibitors of F(1)-ATPase block mitochondrial ATP synthesis in tumor cells [24].

Hydroxy containing flavonoids (quercetin) inhibit xanthine oxidase (PDB code 3NVY): The cause of gout is the deposition of uric acid crystals or its monosodium salt in human joints, which causes joint inflammation and pain. The enzyme, xanthine oxidase, catalyzes the hydroxylation steps of hypoxanthine to uric acid (xanthine is an intermediate). Natural flavonoids with 7-hydroxy groups have been found to bind tightly to xanthine oxidase and are thus current lead compounds for rational drug design as xanthine oxidase inhibitors. The inhibitory potency of the flavonoids towards xanthine oxidase is in the micromolar range [25]. In the crystal structure of quercetin and xanthine oxidase, the inhibitor benzopyran group is between Phe914 and Phe1009, and the B ring is pointed toward the solvent channel leading to the molybdenum binding site (active site). As seen in several studies, the hydrophobic interactions of the conjugated 3 ring structure of quercetin and the active site residues, along with specific hydrogen bonding interactions of hydroxyl groups outside the ring of quercetin (7-hydroxy groups), which interact with Arg880 and Glu802 of the enzyme, promote tight binding. The tight binding of quercetinin, the xanthine oxidase enzyme crystal structure, provides structural evidence that flavonoid-type inhibitors of this enzyme are useful for the treatment of gout and other inflammatory disease states [25]. In Figure 7A bovine xanthine oxidase is shown complexed with Flavin-Adenine Dinucleotide (FAD) in PDB code 3NVY, and Figure 7B shows quercerin (QUE) and MTE (See Table 1 for IUPAC name). Interestingly, in docking studies in the presence of FAD, a modified molecule combining QUE and MTE "moieties" showed very convincing binding in the same pocket as shown in Figure 7B. The structural or chemical features of flavonoids that may be responsible for antioxidant properties may be the conjugated ring system which is resonance stabilized or the presence of certain hydroxyl groups that may exist in different tauntomeric forms [1].

Part II: Understanding flavonoid biosynthesis in order to biomanufacture flavonoids for drug development

Insights to engineering novel flavonoids for drug development (PDB code 1FP2): S-adenosyl-l-methionine (SAM) dependent plant natural product methyltransferases involved in *Medicago sativa* (alfalfa) metabolism have been extensively studied to understand pathways for flavonoid synthesis. Chalcone O-methyltransferase (ChOMT) and isoflavone O-methyltransferase (IOMT) are SAM dependent methyltransferases whose crystal structures with flavonoids have been determined [26]. The crystal structure of ChOMT in complex with the product S-adenosyl-l-homocysteine and the substrate isoliquiritigenin (4,2',4'-trihydroxychalcone) as well as the crystal structure of IOMT in complex with the products S-adenosyl-l-homocysteine and isoformononetin (4'-hydroxy-7-

methoxyisoflavone) have been determined. Both OMT structures yield evidence of a new oligomerization domain and provide atomic-level details of substrate binding. This research provides the foundation for protocols to harvest OMTs via biomanufacturing by elucidating a "structural understanding of substrate specificity of the large family of OMTs." The ultimate goal is to create molecule libraries for an extensive class of natural product biosynthetic enzymes through biological engineering [26].

A molecular understanding of flavonoid biosynthesis: chalcone synthase (PDB code 1CGK): In order to better understand the natural biochemical pathways to become more efficient to synthesize flavonoids (*in vitro*), structural details of enzymes that synthesize flavonoids have been studied. More specifically, in plants, flavonoids phytoalexin and anthocyanin pigments are naturally synthesized as anti-microbial agents. The enzyme chalcone synthase (CHS) produces chalcone, an intermediate leading to phytoalexins and anthocyanins, by a condensation reaction of coumaroyl and malonyl-co enzyme A thioesters (1:3). In the last step of this reaction, the thioesters are converted to a polyketide intermediate that cyclizes. This process, mediated by CHS, is a control point in the biochemical pathway to produce the phytoalexins and anthocyanin pigments. The crystal structure of the CHS-substrate and CHS-product analogues were critical to determine the sequence of decarboxylation and condensation reactions that occur in this step of the pathway. The mechanism of the cyclization reaction leading to chalcone synthesis was elucidated from these structures. In addition, the CHS-resveratrol complex has provided insight into how a related enzyme stilbene synthase uses the same substrates in a different pathway to synthesize resveratrol. Through database searches of sequences similar to CHS, combined with 3D structure analysis, many similar proteins with novel substrate/product specificity and similar mechanisms may be identified. This approach provides a framework for engineering CHS-like enzymes to produce new products for testing for drug development [28].

A molecular understanding of flavonoid biosynthesis: chalcone isomerase (PBD codes 1FM7 and 1JEP): The intramolecular cyclization of bicyclic chalcones into tricyclic (S)-flavanones is catalyzed by chalcone isomerase (CHI) [28]. The activity of CHI is critical for the biosynthesis of flavanone precursors of floral pigments and phenylpropanoid plant defense compounds. Comparisons of CHI complexed with the products 7,4'-dihydroxyflavanone, 7-hydroxyflavanone, and 4'-hydroxyflavanone (PDBs 1FM7 and 1JEP) demonstrated that the 7-hydroxyflavanones all share the same binding site, whereas 4'-hydroxyflavanone binds in an altered orientation at the active site [28]. "Functional and structural studies show that CHI speeds up the intramolecular cyclization of chalcones into biologically active (2S)-flavanones by selectively binding ionized chalcone in a conformation conducive to ring closure in a diffusion-controlled reaction" [28].

Dihydroflavonol 4-reductase (DFR): a key enzyme in the biosynthetic pathway for flavonoid biosynthesis (PDB codes 3BXX and 3C1T): "Dihydroflavonol 4-reductase (DFR) is a key enzyme of the flavonoid biosynthesis pathway which catalyzes the NADPH-dependent reduction of 2R,3R-trans-dihydroflavonols to leucoanthocyanidins. The leucoanthocyanidins are the precursors of anthocyanins and condensed tannins, two major classes of phenolic compounds that strongly influence the bioorganic medicinal properties of wine. DFR has been investigated in many plant species, structural properties were unknown until the three-dimensional structure of the *Vitis vinifera* enzyme complexed with NADP (+) and its natural substrate dihydroquercetin (DHQ) were solved" [29]. Crystals of DFR-NADP (+)-flavonol (myricetin and quercetin) complexes were obtained and their structures solved in order to better understand substrate specificity. Structural complexes of DFR and flavonoids myricetin and quercetin showed significant changes with respect to that of the previously reported DFR-NADP (+)-DHQ complex. "Two flavonol molecules bind to the catalytic site in a stacking arrangement and alter its geometry" [29]. The stacking of the flavonoids myricetin and quercetin in the active site may inhibit enzymatic activity. "The X-ray structures of both DFR-NADP (+)-myricetin and DFR-NADP (+)-quercetin have been deposited and published together with preliminary spectroscopic data" [29]. The structural complexes demonstrate that "flavonols could be inhibitors of the activity of DFR towards dihydroflavonols" in a feedback inhibition mechanism [29].

Understanding biosynthesis of anthocyanins and condensed tannins

Dihydroflavonol reductase (DHF) PDB code 2C29: "The nicotinamide adenine dinucleotide phosphate (NADPH)-dependent enzyme dihydroflavonol 4-reductase (DFR) catalyzes a late step in the biosynthesis of anthocyanins and condensed tannins, two flavonoid classes of importance to plant survival and human nutrition" (30). The crystal structure of *Vitis vinifera* DFR, was determined for detailed structure-function studies. "The 3D structure of the ternary complex obtained with the oxidized form of nicotinamide adenine dinucleotide phosphate (NADP+) and dihydroquercetin, one of the DFR substrates, presents common features with the short-chain dehydrogenase/reductase family. More specifically, the N-terminal domain adopts a Rossmann fold and a variable C-terminal domain, which participates in substrate binding. The NADP/dihydroquercetin/DFR complex confirms the importance of the 131-156 region, which lines the substrate binding site and helps define the role of a specific residue at position 133 (Asn or Asp), assumed to control substrate recognition. The activity of the wild-type enzyme and its variant N133D has been quantified *in vitro*, using dihydroquercetin or dihydrokaempferol. Results demonstrate that position 133 cannot be solely responsible for the recognition of the B-ring hydroxylation pattern of dihydroflavonols" [30]. This work may aid in future experiments for biomanufacture of anthocyanins and tannins via metabolic engineering [2,30].

UDP-glucose: flavonoid 3-O-glycosyltransferase complex (VvGT1) synthesizes anthocyanins (PDB codes 2C1Z and 2C9Z): Anthocyanins are health promoting natural compounds that are responsible for the color of many plant flowers and fruits. In addition, anthocyanins are the precursors of many pigmented polymers in red wine. [31] The red grape enzyme UDP-glucose, flavonoid 3-O-glycosyl transferase (VvGT1), synthesizes the anthocyanins and has shown activity towards many flavonoids. VvGT1 is non-specific with respect to donor-sugar specificity. Since glycosylation is critical to cellular metabolism and VvGT1 is a member of a large multigene family of glycosyltransferases, understanding this enzyme's mechanism is valuable to biotechnology to synthesize flavonoid derivatives efficiently. The 3D structures of VvGT1 was determined in complex with UPD glucose-derived sugar and acceptor kaempferol and in complex with UDP and quercetin. By utilizing both structural and kinetic analysis, a strong foundation for understanding the mechanism of these glycosyl transferases has been established [31].

Structural studies of glycosyltransferase aid in enzyme engineering for bio catalysis (PDB code 3HBF): "The glycosyltransferase UGT78G1 from *Medicago truncatula* catalyzes the glycosylation of various (iso)flavonoids such as the flavonols kaempferol and myricetin,

the isoflavone formononetin, and the anthocyanidins pelargonidin and cyaniding [32]. It also catalyzes a reverse reaction to remove the sugar moiety from glycosides. The structures of UGT78G1 bound with uridine diphosphate or with both uridine diphosphate and myricetin were determined at 2.1 A resolution, revealing detailed interactions between the enzyme and substrates/products and suggesting a distinct binding mode for the acceptor/product. Comparative structural analysis and mutagenesis identify glutamate 192 as a key amino acid for the reverse reaction. This information provides a basis for enzyme engineering to manipulate substrate specificity and to design effective biocatalysts with glycosylation and/or deglycosylation activity" [32].

Similarly, small molecules that may block metal cofactor binding of the Ecto-5-nucleotidase (CD73), complexed with Baicalin (OXE) in PDB code 4H2B, may enhance binding of OXE at adjacent binding site in a negative allosteric effect (Figure 5). Once again derivatives of flavonols with hydrophilic moieties or in this case secondary amine derivatives may bind tightly to enhance effects as tested in docking studies to be reported in full in future work.

Conclusion

The diversity of structural groups of the flavonoids has rendered these compounds to be a rich source of functional groups with potential as lead compounds for a variety of illnesses. As an anti-cancer agent, flavonoids block the cell cycle and induce apoptosis, disrupting cell division and growth [11]. A wide range of targets for other diseases has also been introduced herein. A growing number of flavonoid-protein complexes have already been deposited in the Protein Data Bank (PDB) and the number continues to increase as researchers look towards natural products such as flavonoids for lead compounds for drug development.

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