Computer aided design for molecular inhibitors

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To my family for their love and support through the years.

To Afke for enriching my life in everyway.

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Contribution of Co-Authors

This thesis includes work that was conducted by the author as well as a manuscript that contains work from other contributors and was co-authored by Nicolas Moitessier.

Chapter 1: The design and synthesis of dipeptide inhibitors of DPP-IV was entirely work of the author. The biological assays were conducted by Dr. Lucienne Juillerat.

Chapter 2: The chapter on bicyclic scaffolds of DPP-IV was entirely the work of the author.

Chapter 3: The chapter on virtual screening contains work from several co-authors. Although I did most of the work, the protein preparation was partially conducted by Christopher Corbeil, and the scripts used to extract the results were created and used by Eric Therrien and Nicolas Moitessier.

Abstract

In-silico methods used to aid the development of therapeutic drugs have gained utility in the recent past and continue to grow in importance. Small molecule drugs for the use in the treatment of type 2 diabetes were designed using computational methods to probe the active site of Dipeptidyl Peptidase IV (DPP-IV) and were subsequently synthesized and tested *in-vitro*. A number of pseudodipeptides formed from derivatives of tryptophan and proline were synthesized. The effects of a tetrazole group on the pyrrolidine of proline were studied, as well as the effect of protecting groups on the activity on DPP-IV. Exploring the effects of rigidifying the active dipeptides was next explored by attempting to synthesize the analogous bicyclic structures.

In order to add to the knowledge in the realm of computation tools used for drug discovery, eight docking programs were used to screen a subset of a small molecule database, the Database of Useful Decoys (DUD). This study focused on the effects of protein flexibility, crystallographic waters and program/protein dependence on active compound identification accuracy. This knowledge gained on the efficacy of current docking programs in VS campaign on real world therapeutic targets will allow for more efficient drug design in the search for new therapeutic agents.

Résumé

Les méthodes *in-silico*, utilisées pour faciliter le développement de composés thérapeutiques, ont dernièrement vu leur utilité croître en considérablement. Au cours de ce travail, une série de petites molécules visant le traitement du diabète de type 2 a été conçue en se servant de méthodes informatiques pour sonder le site actif de Dipeptidyl Peptidase IV (DPP-IV) et a, par la suite, été synthétisée et testée *in-vitro*.

Tout d'abord, de nombreux pseudo-dipeptides construits à partir de dérivés de tryptophane et proline ont été préparés. Les effets sur l'activité biologique d'un groupe tétrazole sur le groupe pyrrolidine de la proline ont été étudiés, ainsi que l'effet de la protection des groupes fonctionnels. L'étude de l'impact de la rigidification de ces dipeptides actives a ensuite été envisagée et une synthèse de structures bi-cycliques debutée.

Dans le but d'accroitre les connaissances sur les outils informatiques servant pour la découverte de médicaments, huit programmes d'amarrage ont été évalués sur une banque de données d'une petites molécules, "Database of Useful Decoys" (DUD). Nous nous sommes plus particulièrement intéressés à l'impact de la flexibilité des protéines, de l'eau cristallographique et du type de programme utilisé sur la fiabilité des résultats. Les données collectées au cours de cette étude va nous permettre de développer des programmes plus efficaces et par la suite permettre une meilleure fiabilité de ces programmes dans les campagnes de criblage virtuel futures qui auront pour but de trouver de nouveaux agents thérapeutiques.

List of Abbreviations

Å	Ångström
ANOVA	analysis of variance
Ar	aryl
AUAC	the area under the accumulation curve
AU-ROC	Area under the receiver operating characteristic
BED-ROC	Botlzmann-enhanced discrimination of receiver operating characteristic;
CDK2	cyclin-dependent kinase 2
BPB	Benzylprolyl-(*S*)-2-[*N*-(*N*′-benzylprolyl)amino]benzophenone
Boc	*tert*-butoxycarbonyl
Bs	broad singlet
Cbz	benzyloxycarbonyl
DCM	dichloromethane
de	diastereomeric excess
DMF	dimethylformamide
DMSO	dimethyl-sulfoxide
DPP-IV	dipeptidyl peptidase-IV
DUD	directory of useful decoys
E^+	electrophile
EF	enrichment factor
eHiTS	electronic High Throughput Screening
ER	estrogen receptor
ESI-MS	electrospray ionization mass spectrometry
Et_2O	diethyl ether
Et_3N	triethylamine
EtOAc	ethyl acetate
FITTED	flexibility induced through targeted evolutionary description
FMOC	9*H*-fluoren-9-ylmethoxycarbonyl
GA	Genetic Algorithm
GIP	gastric inhibitory polypeptide
GLIDE	Grid-based Ligand Docking with Energetics
GLP-1	glucagon-like peptide 1
GOLD	Genetic Optimization for Ligand Docking
HATU	2-(7-Aza-1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
HIVRT	HIV reverse transcriptase
HRMS	High Resolution Mass Spectrometry
HTVS	High throughput Virtual Screen
HIVP	HIV protease
J	coupling constant
LGA	Lamarckian Genetic Algorithm
P38	mitogen activated protein
m/z	mass-to-charge ratio
MeCN	acetonitrile

MeOH	methanol
NMR	Nuclear Magnetic Resonance
Nu	nucleophile
PBAB	(2-[N-(a-picolyl-amino]benzophenone)
PDB	protein database
q	quartet
Rf	the retention factor
RIE	the robust initial enhancement;
RM	reaction mixture
ROC	the area under the receiver operating characteristic curve
RT	room temperature
s	singlet
SA	simulated annealing
t	triplet
TBAF	tetrabutylammonium flouridefluoride
TBS	*tert*-butyldimethylsilyl
TK	thymidine kinase
VS	virtual screening
δ	chemical shift

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Chapter 1: DPP-IV as a target for the treatment of Type 2 Diabetes

1.1 Introduction

Type 2 diabetes is a disease in which the body fails to use insulin properly, combined with relative insulin deficiency. Acute complications include hypoglycaemia and diabetic ketoacidosis while long-term complications include cardiovascular disease, chronic renal failure, and retinal damage (Rotella 2004; Weber 2004). Injections of insulin provide an effective but inconvenient treatment for the disease. Herein, a treatment involving the indirect control of insulin is described.

The incretin hormones GLP-1 and glucose-dependent gastric inhibitory polypeptide (GIP) are released by the small intestine in a response to the intake of food. GLP-1 and GIP both regulate insulin secretion in a glucose-dependent manner. The many roles of GLP-1 within the human body include stimulation of insulin secretion and biosynthesis, inhibition of glucagon release, slowing gastric emptying and reducing appetite. However, the positive effects of endogenous GLP-1 and GIP are limited because they have very short plasma half-lives. The short plasma half-lives are due to rapid enzymatic deactivation. DPP-IV activity is so effective that the half-life of GIP is approximately 7.3 minutes (Deacon, Nauck et al. 2000) and GLP is a mere 1-2 minutes (Vilsboll, Agerso et al. 2003). The enzyme responsible for the metabolism of these incretin hormones is DPP-IV. By finding efficient inhibitors of DPP-IV the levels of endogenous GLP-1 and GIP can be controlled, thereby providing an effective treatment for type II diabetes.

Dipeptidyl peptidase-IV (DPP-IV), also known as ADA and CD26 is an antigenic enzyme expressed on the surface of most cell types and is an intrinsic membrane glycoprotein and a serine exopeptidase which has a preference to cleave X-proline dipeptides at the N-terminus of polypeptides. While substrate specificity studies show DPP-IV's preference for cleavage of peptides containing a proline residue in P_1, interestingly, GLP-1 and related glucagon family members contain alanine at this position. DPP-IV plays an important role in glucose metabolism as it is responsible for the degradation of incretins such as glucagon-like peptide 1 (GLP-1)(Weber 2004).

In an effort to obtain a more complete understanding of the mechanism of inhibition of DPP-IV and to develop potent inhibitors of DPP-IV, computational methods were used to design potential inhibitors, which were then synthesized and subsequently tested *in-vitro*.

An account of why DPP-IV was chosen as a target for the treatment of type 2 diabetes will follow, leading into the computer-aided design and synthesis of DPP-IV inhibitors.

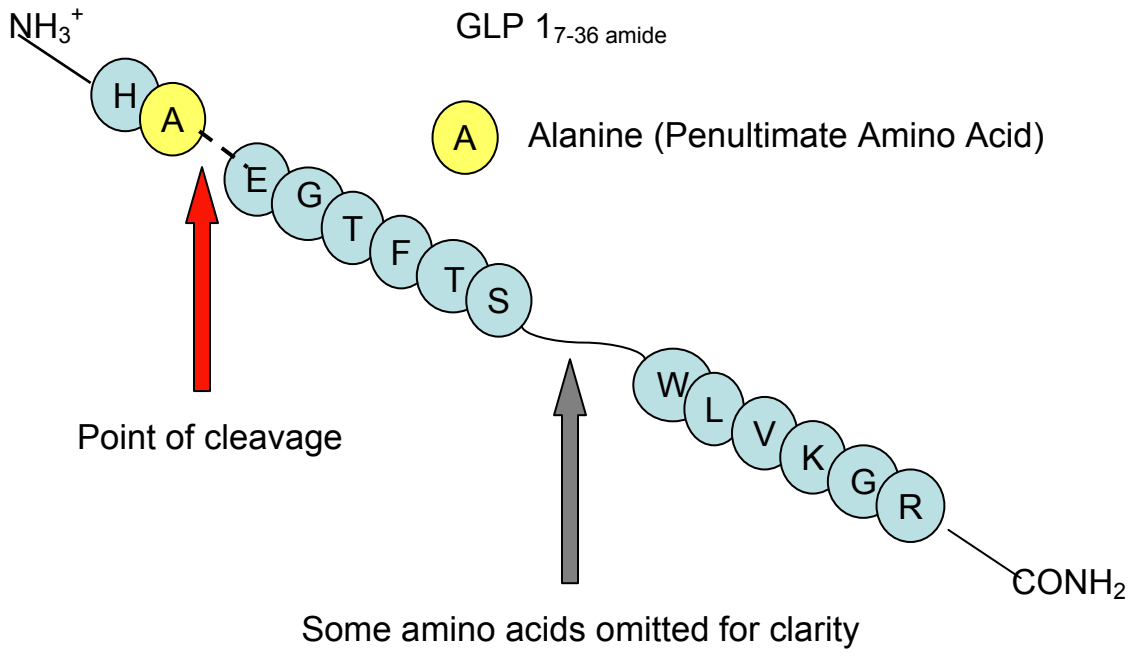


Figure 1.1 Point of cleavage for GLP-$1_{7\text{-}36}$ amide by DPP-IV

1.2 The incretin effect

Regulation of plasma glucose levels are controlled by a complex set of signals from the brain and gut that initiates pancreatic hormone release. In patients who suffer from type 2 diabetes, the response to glucose stimulation is defective. Type 2 diabetes is characterized by insulin resistance and diminished insulin action (Campbell 2007). Pancreatic α-cell function is also impaired in type 2 diabetes, which leads to excess secretion of glucagon.

Healthy individuals maintain a plasma glucose level of 140 mg/dL or less (2006). This level is maintained throughout variations in food intake due in large part to pancreatic β-

cell secretion of insulin. This release of insulin is in response to the intake of food, and has been correlated to the secretion of insulinotropic hormones GLP-1 and GIP and their effects on glucagon and insulin secretion from pancreatic islet α and β-cells. Studies which compared the effect of oral ingestion with intravenous administration of glucose indicate that insulin response to glucose is more pronounced when glucose is transported through the gut rather than a direct administration to the bloodstream (Nordio, Lamedica et al. 1964). Due to the correlative effect of incretin secretion on insulin secretion, this augmentation of insulin secretion resulting from orally obtained glucose has become known as the incretin effect. The incretin effect is either absent or significantly diminished in individuals with diabetes.

GLP-1 and GIP are considered the primary sources of stimuli for β-cell insulin secretion. These incretins have a considerable amino acid homology and in plasma, they are both rapidly inactivated by the serine protease DPP-IV. Secretion of GLP-1 and GIP is coupled with nutrient absorption and therefore on ambient glucose levels. The functional dependence of the incretin effect serves as the mechanism for guarding against hypoglycemia.

The loss of GIP action has been shown to lead to decrease glucose-dependent insulin secretion from the intake of food, and also leads to mild glucose intolerance in response to oral glucose administration. GLP-1 has been shown to be a significant part of insulin response to the oral intake of glucose and essential for normal glucose tolerance (Knudsen 2004; Weber 2004). While GIP and GLP-1 both have insulinotropic potential in healthy individuals, GIP loses most of its insulinotropic potency in individuals with type 2 diabetes.

1.3 Rational for DPP-IV inhibition to improve incretin activity in Type 2 Diabetes

Several clinical studies have shown that infusion of exogenous GIP and GLP-1 can be used to control the levels of glucose in individuals with type 2 diabetes. It has been shown that patients treated with GLP-1 also experienced delayed gastric emptying,

decreased body weight and improvements in insulin sensitivity and β-cell function. This kind of evidence has been used to show that controlling levels of endogenous incretins offers an effective means for the reduction of plasma glucose elevations in cases of type 2 diabetes. Figure 1.2 illustrates the path of by which the metabolic cleavage of GLP-1 and GIP can be mediated by inhibitors of DPP-IV thereby increasing insulin release and subsequently lowering plasma glucose levels.

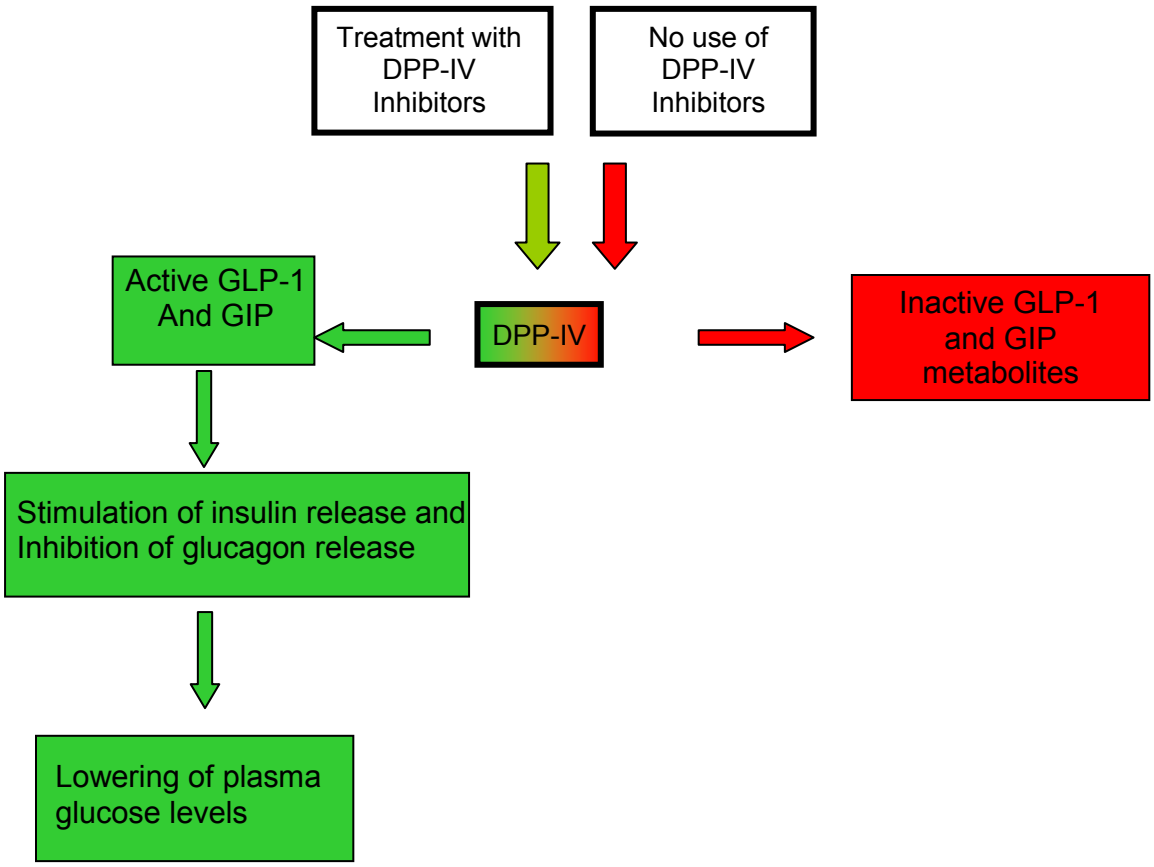


Figure 1.2 Diagram illustrating DPP-IV inhibition for controlling glucose levels

The use of native GLP-1 supplementation in the treatment of type 2 diabetes is not a viable solution due to its quick deactivation by DPP-IV. DPP-IV is a member of the serine protease family which recognizes amino acid sequences which possess proline or alanine at the second position from the N-terminal. It is a highly specific serine protease which rapidly cleaves both GLP-1 and GIP (Figure 1.1). DPP-IV inhibitor therapy is therefore based on blocking the action of DPP-IV by using a molecule that effectively

competes for the binding site in DPP-IV. This mechanism therefore prolongs the time that endogenous GLP-1 exists in plasma by reducing enzymatic deactivation.

These biological effects of DPP-IV show that inhibition of the enzyme would lead to increased serum levels of GLP-1. In fact, animal studies have shown that inhibition of DPP-IV leads to increased glucose tolerance, delayed onset of diabetes and increased insulin sensitivity (Pederson, White et al. 1998).

The previously described effects of controlling the levels of the aforementioned incretins provided the impetus for studying DPP-IV as an orally available treatment for type 2 diabetes. At the time that this study was started, there were no DPP-IV inhibitors on the market. However, at the time of this writing, a number of DPP-IV inhibitors have passed clinical trials and entered the market. The first inhibitor on the market was sitagliptin (by Merck & Co.), which was then followed by the structurally similar vildagliptin (by Novartis) and saxagliptin (by Bristol-Myers Squibb and AstraZeneca).

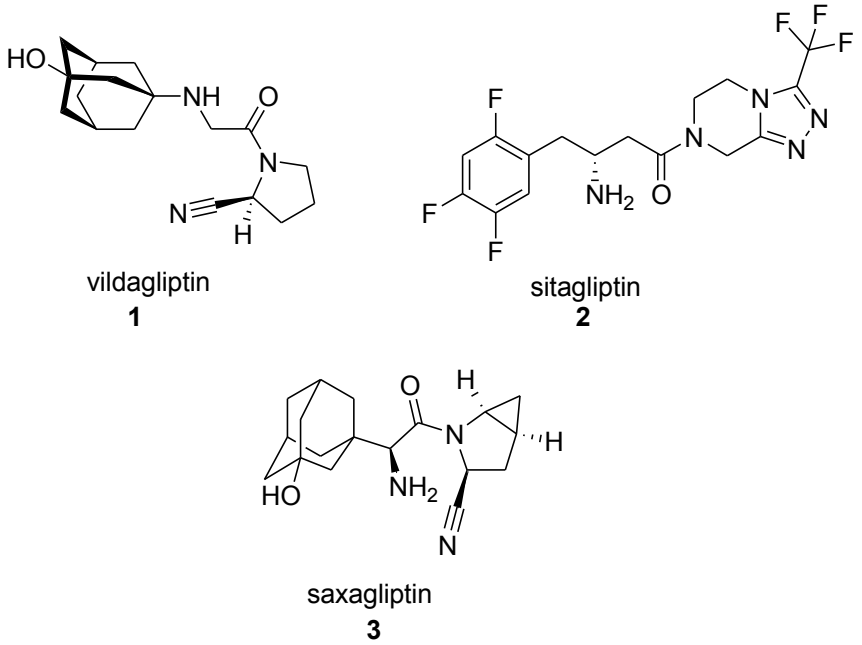


Figure 1.3 DPP-IV inhibitors currently on the market

1.4 Rational for design of tetrazole-based inhibitors

Our own work in this area led to the design and synthesis of a number of potential inhibitors. The initial series of inhibitors were designed using computational methods and was based on the dipeptide inhibitor derived from **4** (*S*)-2-cyanopyrrolidine, shown in its protonated state (**5**) as shown in Figure 1.4.Compound **5** had been reported as a reversible and potent inhibitor (Sakashita, Kitajima et al. 2005). This inhibitor contains a (*S*)-2-cyanopyrrolidine which acts as a proline mimic at the P_1 site and at the P_2 site an aliphatic amino acid with an amino group which is important in binding to the enzyme. The nitrile group plays a very crucial role by forming a covalent bond with the serine in the DPP-IV binding pocket.

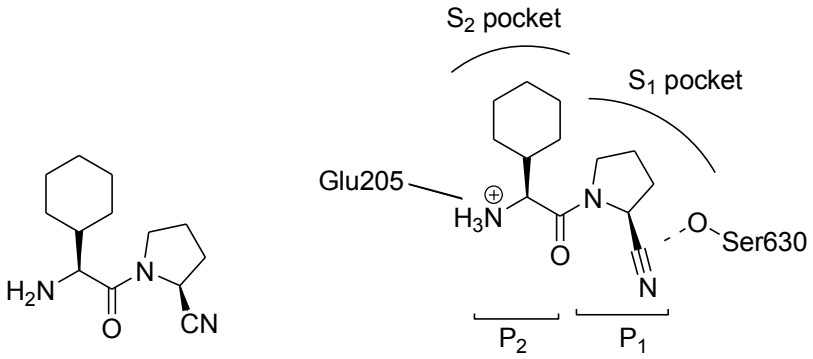


Figure 1.4 DPP-IV inhibitor showing binding

A problem associated with this family of nitrile based inhibitors is that they can possess a basic chemical instability due to the *cis*-rotamer **7** being prone to intramolecular cyclization leading to the inactive bicyclic compound **8** (Figure 1.5). This provided the impetus to develop compounds that were not inclined to undergo this type of intramolecular cyclization. The replacement of the nitrile group with a tetrazole is discussed later in this chapter.

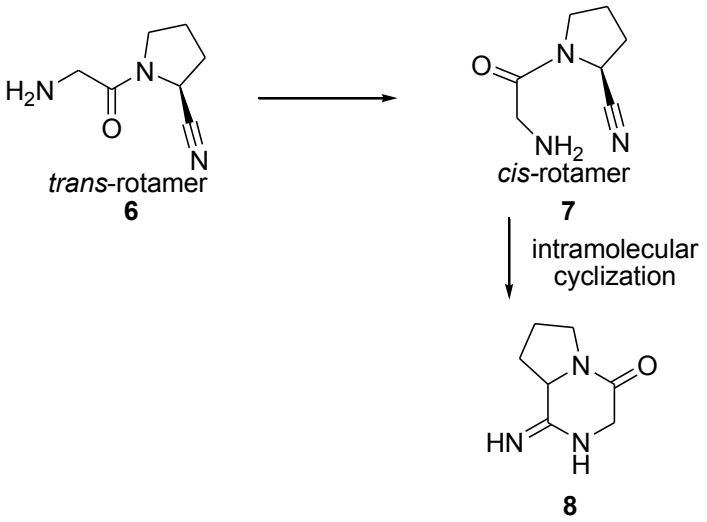


Figure 1.5 Intramolecular cyclization of nitrile based inhibitors

1.5 Docking Results and methods used in the design of DPP-IV inhibitors

Using the DPP-IV protein structural data available from the PDB a number of potential dipeptide inhibitors were docked using the Maestro suite. Based on the docking study a series of dipeptide inhibitors of DDP-IV were made and tested *in vitro* as shown in Figure 1.6.

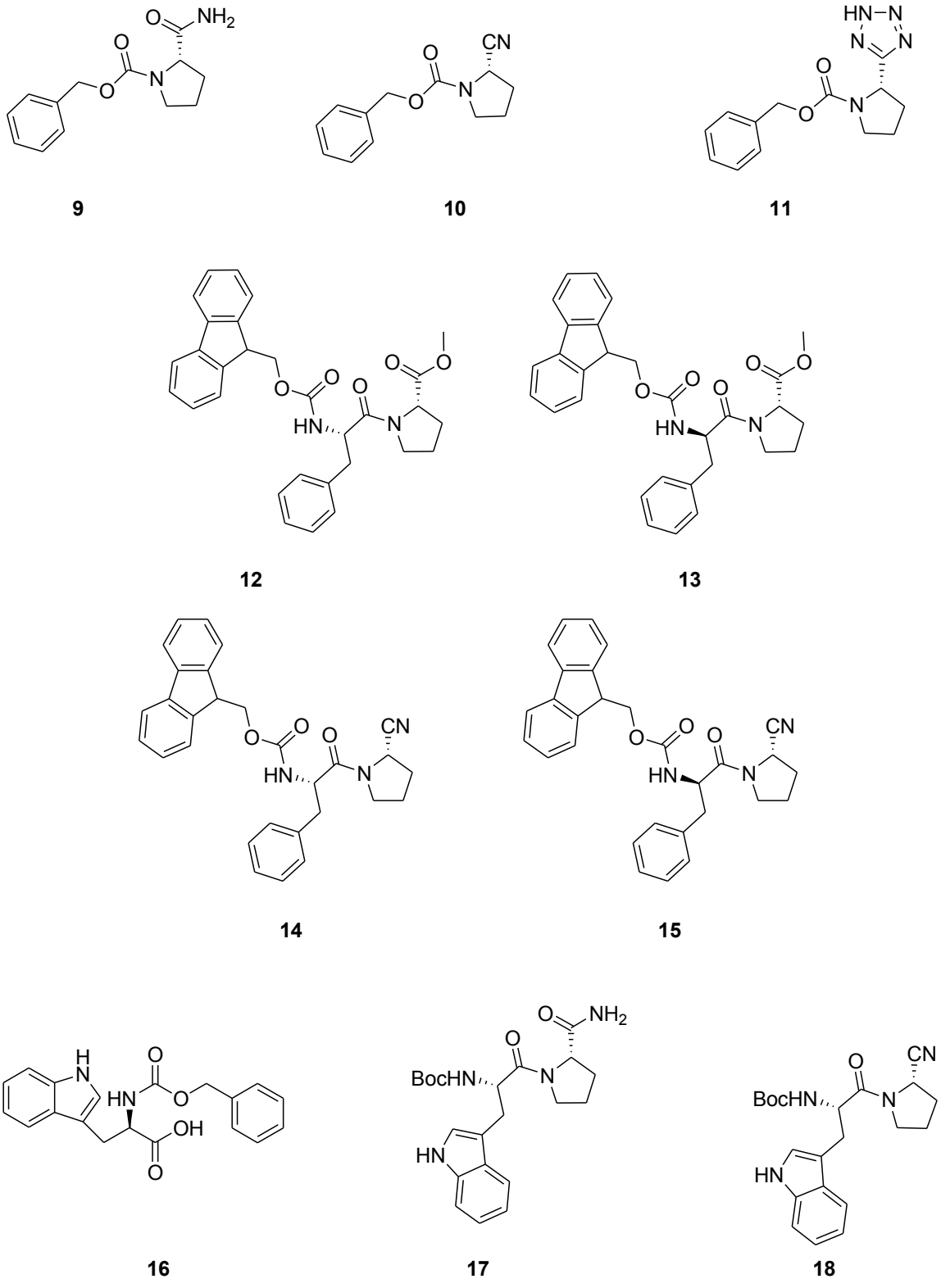
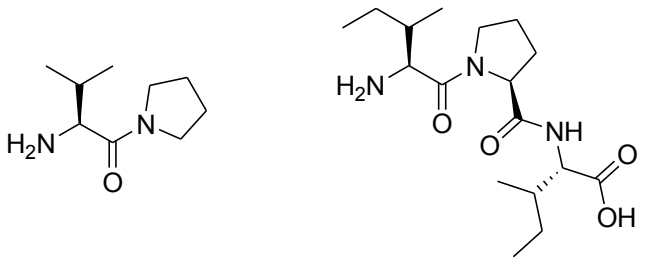


Figure 1.6 Potential DPP-IV inhibitors synthesized

A review of the current literature of DPP-IV inhibitors was conducted at the beginning of the study, which revealed that the vast majority of inhibitors of DPP-IV were pyrrolidine or thiozolidine derivatives (proline mimetics), often containing an electrophilic group and a primary amine. Some examples are valine-pyrrolidide and diprotin A (Figure 1.7).



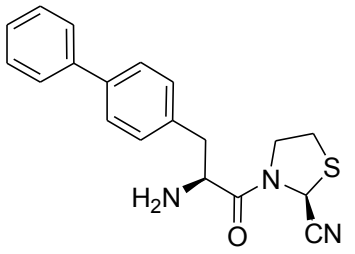
19 **20**

Figure 1.7 Valine-pyrrolidine and Diprotin-A

Despite the existence of a number of potent inhibitors of DPP-IV that were neither based on pyrrolidine nor thiozolidine moieties, it was thought that a good starting point would be based on the elaboration of the pyrrolidine derivatives. The importance of finding completely novel scaffolds that diverge from the pyrrolidine ring cannot be ignored, but it was thought that for the first round of screening it would be important to iron out the details of the docking process. In addition, novel interactions between key residues could still be explored using the pyrrolidine derivatives, which could then be applied to future novel chemical series.

The binding site of DPP-IV can be considered to be small but due to binding induced flexibility, may fit larger groups than initially thought. Quai et al. probed the size requirements of the S2 pocket and discussed novel biaryl-based DPP-IV inhibitors that are based on the pyrrolidine structure (PDB ID: 2AJL), which lose activity as soon as the linear biphenyl group is replaced by a larger, conformationally hindered napthalen-2-yl group Figure 1.8 (Qiao, Baumann et al. 2006). The large biaryl group fills a large cavity in the binding site and is also capable of favourable π-stacking interactions, sterics can have a very significant impact with groups of this size in the S2 pocket. However, protein flexibility was also evaluated via comparison of the binding site of 2AJL and another x-

ray crystal structure co-crystallized with a ligand that does not fill this part of the binding site (eg. PDB ID: 2BGN) revealed that the Arg358 residue undergoes significant movement, in order to make space for the large biaryl group (Weihofen, Liu et al. 2005). The crystal structure of 2AJL showed that there was more space in this part of the binding site than previously thought. This suggested the possibility for the placement a functionalized bicyclic ring in this large pocket in order to target some key interactions in this region of the binding site.



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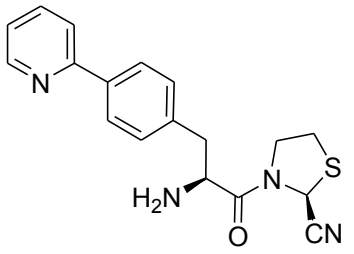
K_i = 0.36 μM



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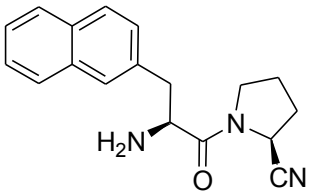
K_i = 0.355 μM



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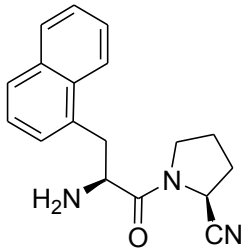
K_i = 8.9 μM



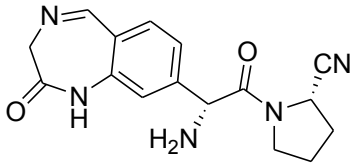
24

K_i =>12.8 μM

Figure 1.8 K_i values of selected 4-substitued phenylalanine cyano-(*S*)-pyrrolidine derivatives from study by Qiao et al. (Qiao, Baumann et al. 2006)

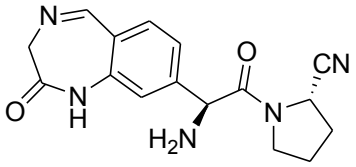
A small set of potential inhibitors based on the pyrrolidine structure was drawn in Chemdraw in order to dock the compounds *in-silico*. Functionalized benzimidazoles, tetrazoles and benzodiazepines were incorporated into this small test set. Small sets of related ligands were docked in the docking program Glide which is further discussed in Chapter 3. Glide outputs a numerical score (G-Score) for each of the ligands that have been docked, with the lowest G-Scores corresponding to what it predicts to be the best binding ligands. These sets consisted of groups such as benzimidazoles, benzodiazepines

and indoles as the scaffolds which were then functionalized at a number of different positions based previously published SAR data (Weber 2004; Sakashita, Kitajima et al. 2005; Qiao, Baumann et al. 2006). The set was docked using the crystal structure obtained from the PDB (PDB ID: 1WCY). Some selected results are shown below (Figure 1.9), with the corresponding G-scores for the 1st ranked poses.



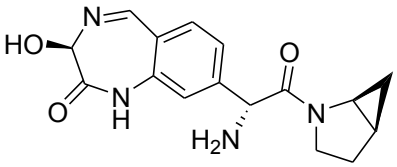
G-Score: -7.4

25



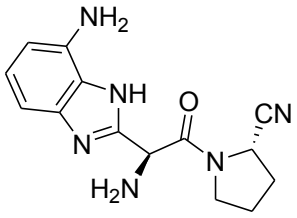
G-Score: -12.4

26



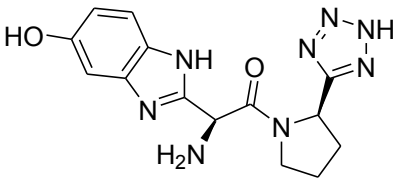
G-Score: -8.2

27



G-Score: -12.7

28



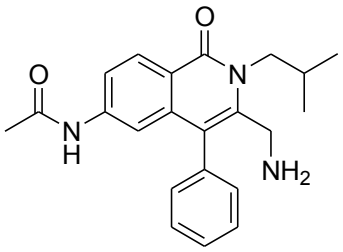
G-Score: -9.6

29

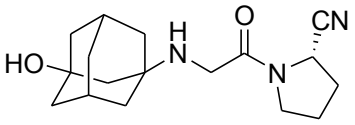
Figure 1.9 Selected structures docked into the active site of 1WCY using Glide

In order to get a point of reference, several published inhibitors were docked into the DPP-IV crystal structure obtained from the PDB (PDB ID: 1WCY) using Glide. It was surprising to see that for the most part, the G-Scores were lower than the best structures that were screened from the test set, although compounds which rely on covalent bonding

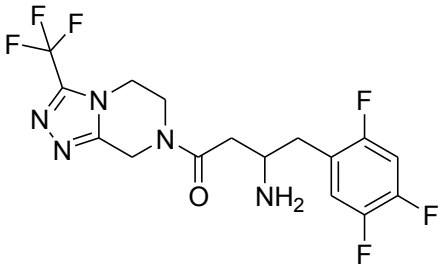
for inhibition were expected to have lower score/true activity ratio due to Glide's inability to consider covalent bonds while docking. A small selection of published inhibitors and their G-Scores are shown below (Figure 1.10). The G-Scores were empirically by modifying the G-Scores of groups containing a proline-nitrile group. By reviewing the literature (Peters, Weber et al. 2004; Weber 2004; Haffner, McDougald et al. 2005; Qiao, Baumann et al. 2006; Vilsboll and Knop 2007) it was determined that compounds possessing a proline-nitrile group should have an empirical value of 4 added to the base G-Score to include the free energy of the covalent bond formed between the protein and ligand upon binding. Further investigation with a larger set of known ligands and proteins would have to be conducted in order to determine whether or not the scoring is representative of the activity of the ligands along with the synthesis and bioassays of the selected compounds.



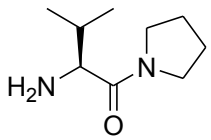
G-Score: -8.8
30



G-Score: -11.2
31



G-Score: -8.5
32



G-Score: -7.1
33

Figure 1.10 Selected G-Scores of published inhibitors of DPP-IV docked into 1WCY

Most of the molecules were minimized using the Macromodel module with the MM3* forcefield prior to docking. However, some of the molecules containing "non-standard"

heterocycles were not recognized by the MM3* forcefield provided in Glide. The series that contained tetrazoles and tricyclic tetrazole containing structures failed to be minimized using MM3*. These structures were successfully minimized using MM2 in Chem-3D prior to docking in Glide.

When the structures that contained the hydroxylated benzimidazole and the tetrazole moieties (Figure 1.9) were docked using Glide, some interesting hydrogen bonds were observed in Glide's Pose Viewer. These interactions are shown in Figure 1.11 and Figure 1.12

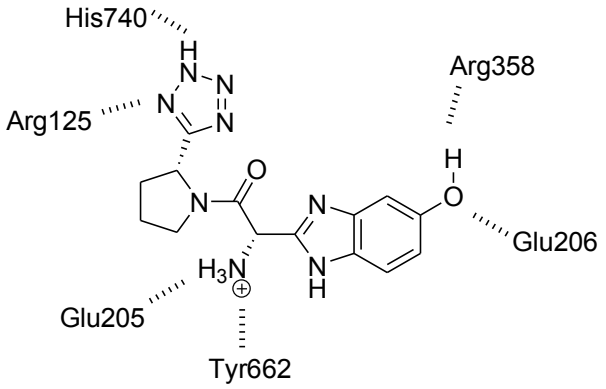


Figure 1.11 Hydrogen bond interaction of potential DPP-IV inhibitor **34** with key residues

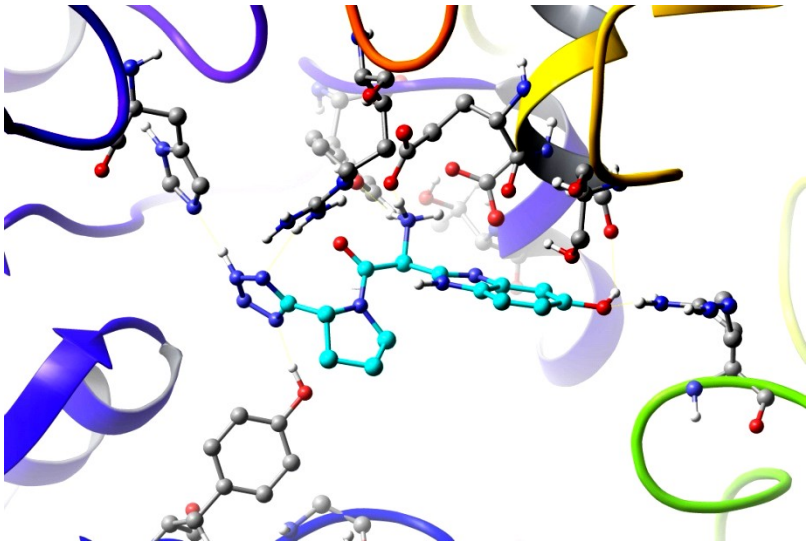
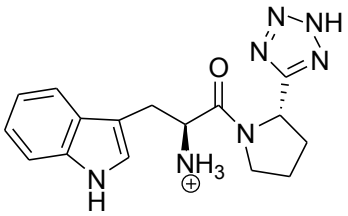


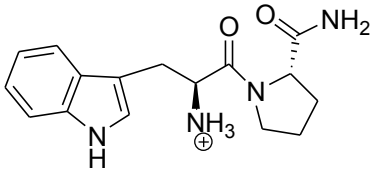
Figure 1.12 Hydrogen bond interactions suggested by Glide between **34** and 1WCY

With this promising structural information in hand, a novel series was designed and docked with their synthetic feasibility in mind. This series saw the replacement of the benzimidazole ring system with an indole ring system. The G-Scores obtained from the Glide docking runs showed a significant improvement over the benzimidazole series. The synthesis of this set of ligands is also expected to be significantly shorter than that of the benzimidazole series, since both enantiomers of tryptophan are commercially available and can be used to quickly form one half of the proposed molecules. Figure 1.13 illustrates selected ligands with the G-Scores of the top 5 ranked poses. The results obtained from Glide did not consider covalent bonds, which are important in compounds that are able to form covalent bonds such as those with a proline-nitrile moiety, however the compounds that scored highly, did show significant hydrogen bond network formation.



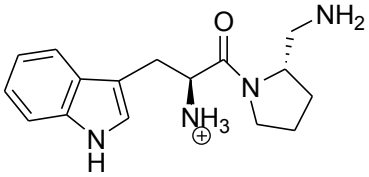
G-Score: -9:2

35



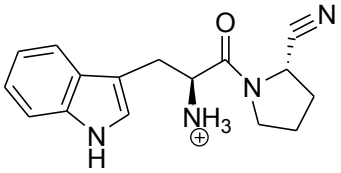
G-Score: -10.1

36



G-Score: -9.4

37



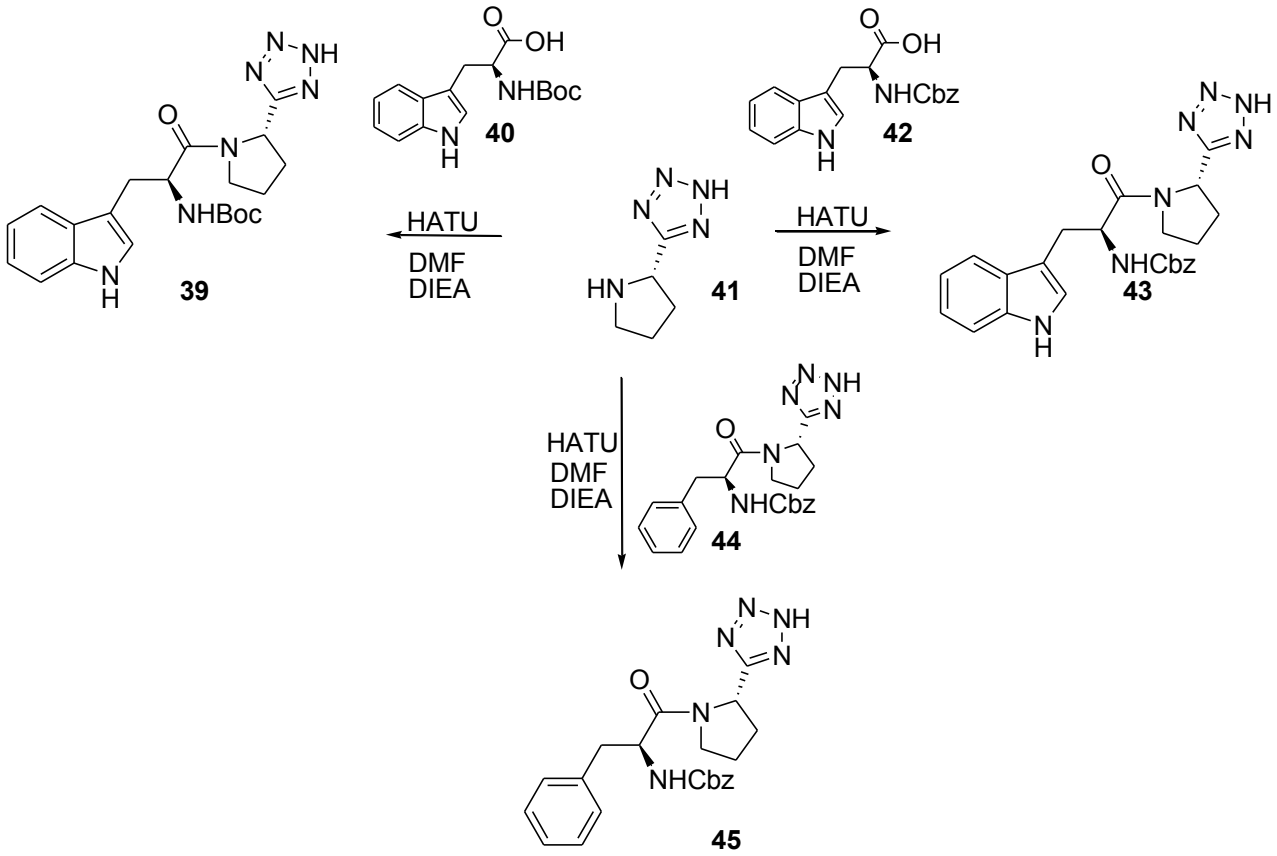
G-Score: -13.3

38

Figure 1.13 Selected indolic ligands, docked into PDB ID: 1WCY

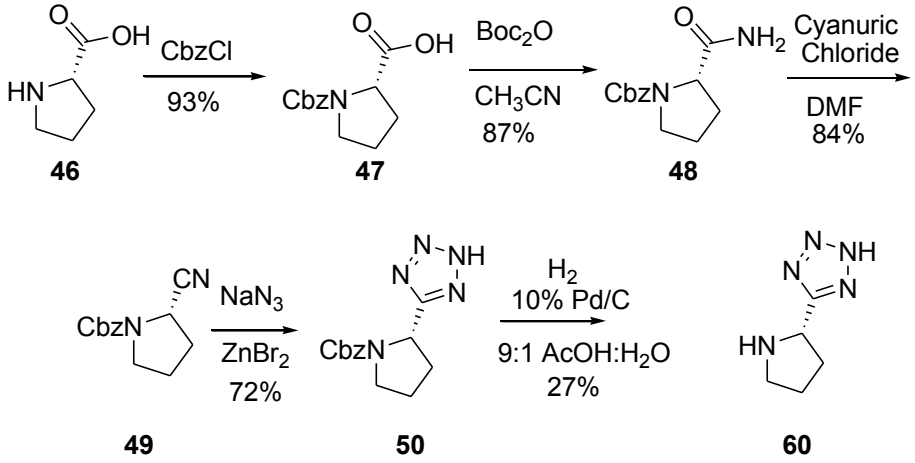
1.6 Synthetic routes to the pyrrolidine building block

The ligands shown above were chosen, because the synthetic pathway to form the desired pyrrolidin-2-yl-tetrazole building block, went through a Cbz protected L-prolinamide structure, as well as a Cbz-L-pyrrolidine nitrile. Simple coupling reactions described in Scheme 2-1 show a synthetic plan in which several of the tetrazole precursors could be used as the right hand side of the ligand using standard coupling methods.



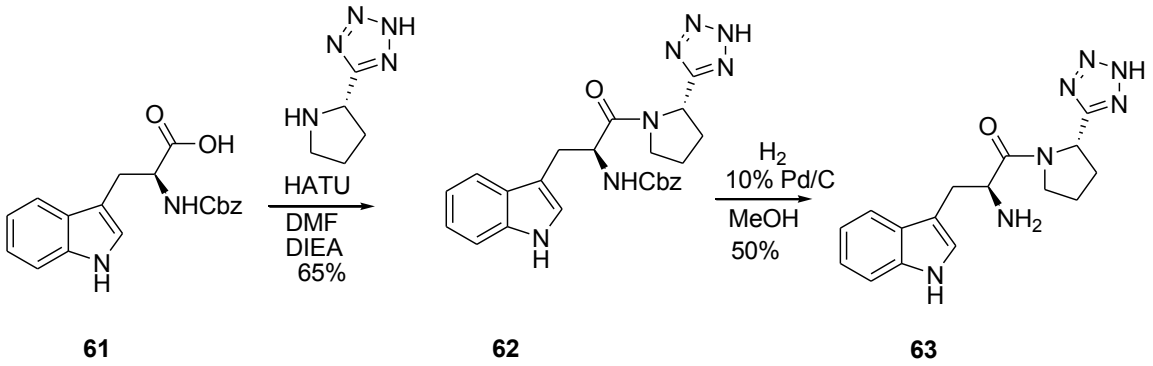
Scheme 1-1 Illustration of the synthetic pathway to **39**, **43**, **45**

The synthesis of the desired tetrazole building block was carried out by previously described methods.(Antti and Per 2005)



Scheme 1-2 Synthetic pathway to pyrrolidin-2-yl-tetrazole for producing a number of tryptophan derivatives.

With the pyrrolidin-2-yl-tetrazole moiety in hand, coupling with various *N*-protected amino acids is possible. Scheme 1-3 shows the coupling that could be carried out of an *N*-Cbz protected tryptophan coupled with the derivatized proline followed by removal of the Cbz group to yield the free amine.



Scheme 1-3 Method for coupling CBZ protected tryptophan with pyrrolidin-2-yl-tetrazole

While the synthetic route which uses Cbz as a protecting group is effective at isolating the deprotected pyrrolidin-2-yl-tetrazole, it is not useful at forming the nitrile analogue. The difficulty lies in the problems observed when attempting to remove the Cbz group in the presence of the nitrile. In order to obtain the nitrile analogue, an alternate protecting group such as Boc was used.

Early DPP-IV inhibitors were found to commonly bind to DPP-IV in a substrate like mode, that is, a mode in which the proline or proline mimic was found to bind into the proline binding pocket. More recent X-ray crystallographic studies showed that an unexpected reverse binding mode was responsible for the activity of some β-phenethylamines (shown in Figure 1.14 developed by Nordhoff *et al.* were binding with the proline moieties outside of the proline binding pocket of DPP-IV (Nordhoff, Cerezo-Gálvez et al. 2006). Figure 1.4 shows valine-pyrrolidide (**19**) in grey, binding in the expected manner and sitigliptin (**2**) in green binding in a reserve fashion. It is thought that the inhibitor **67 (**Figure 1.15**)** which has the pyrrolidin-2-yl-tetrazole may bind in a similar, reverse mode due to the restrictively small size of the proline binding pocket of DPP-IV and the relatively large size of the pro-tetrazole moiety.

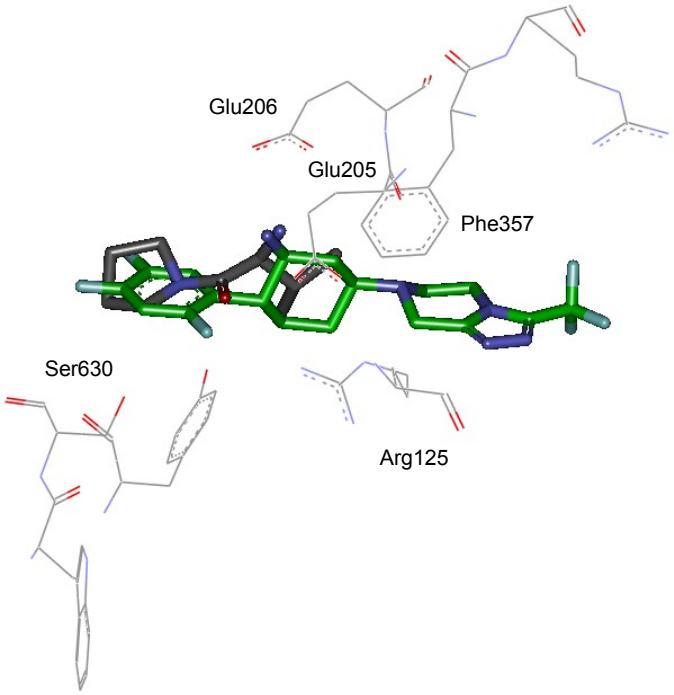


Figure 1.14 Valine-pyrrolidine (**19**) (grey) and sitigliptin (**2**) (green) illustrating reverse binding

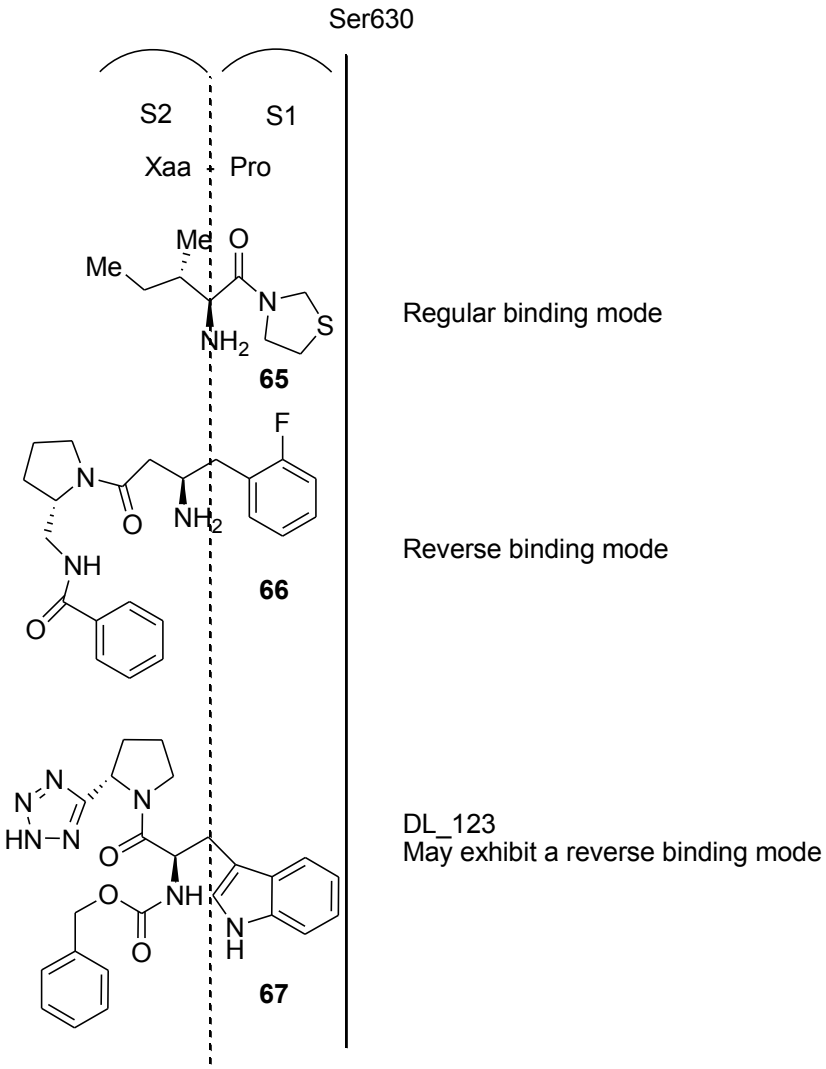


Figure 1.15 Possible reverse binding

1.7 Results

Prolyl-dipeptidyl-aminopeptidase (DPP IV) activity was measured with the fluorogenic substrate Gly-Pro-AMC using the human brain astrocyte-derived LN18, LN229, and LNZ308 glioblastoma cells, the human brain-derived HCEC endothelial cells and the human lung-derived PO03 and skin-derived PG98/5 fibroblasts. The initial inhibition screening experiments were performed using cell extracts, obtained by extracting cell layers in PBS-0.1% Triton X-100 to determine the potential of the developed inhibitors to inhibit total cellular enzyme activities (Lawandi, Toumieux et al. 2009) (Table 1-1 and Table **1-2**).

A number of dipeptides based on proline were synthesized and tested for activity against DPP-IV *in-vitro*. Certain intermediates were sent for testing and were found to show some activity. For example, **11** showed activity, despite being originally designed as an advanced intermediate for a coupling reaction. The activity found in **11** was surprising considering that compound **10** which contained a nitrile at the same position as the tetrazole in **11** showed no activity. The nitrile analogue was expected to be more active due to the nature of binding to the active site of DPP-IV though covalent bonding between the nitrile and Ser630. The tetrazole ring in **11** suggests that a tetrazole group may play an important role in binding to DDP-IV that has not previously been examined. This result warrants further study in which the tetrazole ring would be substituted in place of the nitrile group in compounds that are shown to be active that contain a nitrile.

Another surprise was seen in the activity that was exhibited by **12** and **13**. Both of these compounds had ester moieties in the position where similar DPP-IV inhibitors would have had nitriles. The compounds in which the esters are replaced by nitriles, should show even more activity and is currently under investigation by synthesizing compounds **14** and **15**.

	Gly-Pro-AMC (DPPIV-like)[a]					
	LN18[b]	***LN18 T[b]***	***LN229[b]***	***LN229 T[b]***	***LNZ308[b]***	***LNZ308 T[b]***
9	-	-	-	-	-	-
10	-	-	-	-	-	-
11	+	++	+	++	+	+
12	-	+	+	+	-	-
13	-	+	-	++	-	++
14	In prog	In prog	In prog	In prog	In prog	In prog
15	In prog	In prog	In prog	In prog	In prog	In prog
16	-	-	-	-	-	-
17	-	-	-	-	-	-
18	-	-	-	-	-	-

Table 1-1 Inhibition of activity of DPP-IV endoprotease activity in cell extracts for human glioblastasmo cells

[a] -: no inhibition at 100 μM; +: 10-50% inhibition at 100 μM; ++: > 50% inhibition at 100 μM; +++: > 90% inhibition at 20 μM; nd: not determined; [b] LN18, LN229, LNZ308: human glioblastoma cells; [c] HCEC: human brain-derived endothelial cells; [d] PO03: human lung-derived fibroblasts; PG98/5: human skin-derived fibroblasts.

	Gly-Pro-AMC (DPPIV-like)[a]					
	HCEC[b]	***HCEC T[b]***	***PO03[c]***	***PO03 T[c]***	***PG98/5[c]***	***PG98/5 T[c]***
9	-	-	-	-	nd	nd
10	-	-	-	-	-	-
11	-	+	-	+	nd	nd
12	-	-	nd	nd	nd	nd
13	-	+	-	+	-	-
14	In prog	In prog	In prog	In prog	In prog	In prog
15	In prog	In prog	In prog	In prog	In prog	In prog
16	-	-	-	-	-	-
17	-	-	-	-	-	-
18	-	-	-	-	-	-

Table 1-2 Inhibition of activity of DPP-IV endoprotease activity in cell extracts for human brain-derived endothelial cells; c PO03: human lung-derived fibroblasts; PG98/5: human skin-derived fibroblasts.

[a] -: no inhibition at 100 μM; +: 10-50% inhibition at 100 μM; ++: > 50% inhibition at 100 μM; +++: > 90% inhibition at 20 μM; nd: not determined [b] HCEC: human brain-derived endothelial cells; [c] PO03: human lung-derived fibroblasts; PG98/5: human skin-derived fibroblasts.

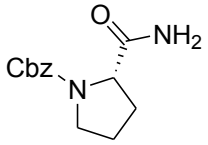
1.8 Conclusion

Dipeptide inhibitors of DPP-IV were designed using computational tools, synthesized and tested for activity *in-vitro*. Two dipeptides (**12** and **13**) showed activity despite possessing an ester at a position in which similar compounds with a nitrile could form an important covalent bond with Ser630 of DPP-IV's active site. This finding suggested that the synthesis of **14** and **15** should be carried out in order to determine whether an increase in activity could be achieved by introducing a nitrile group capable of covalently bonding to the active site of DPP-IV.

Activity was also found in the intermediate compound **11** which contained a tetrazole group at the same position as the nitrile group in compound **10,** which showed no activity in the *in-vitro* testing. This finding suggests that activity may be increased by replacing nitrile groups with tetrazoles at this position on the pyrrolidine ring and is currently under investigation. Other future work will use the knowledge gained from the work on the dipeptide inhibitors and apply them to bicyclic scaffolds described in chapter 2.

1.9 Experimental Section

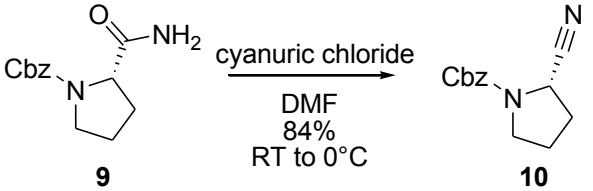
(S)-benzyl 2-carbamoylpyrrolidine-1-carboxylate (9) (Antti and Per 2005)



9

Figure 1.16 (S)-benzyl 2-carbamoylpyrrolidine-1-carboxylate (**9**)

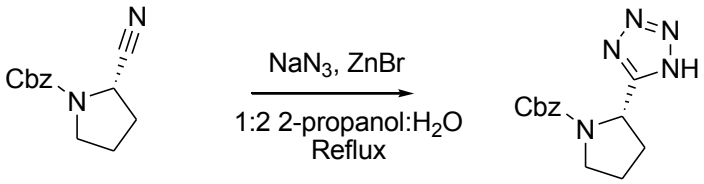
(S)-benzyl 2-cyanopyrrolidine-1-carboxylate (10) (Antti and Per 2005)



9 **10**

Scheme 1-4 Formation of Z-Pro-CN ((S)-benzyl 2-cyanopyrrolidine-1-carboxylate) (**10**)

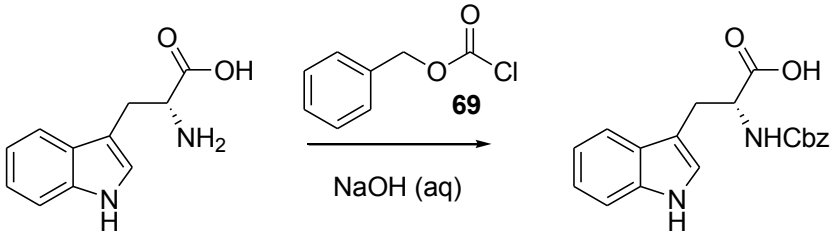
(S)-benzyl 2-(1H-tetrazol-5-yl)pyrrolidine-1-carboxylate) (**11**) (Antti and Per 2005)



10 **11**

Scheme 1-5 Formation of (S)-benzyl 2-(1H-tetrazol-5-yl)pyrrolidine-1-carboxylate) (**11**)

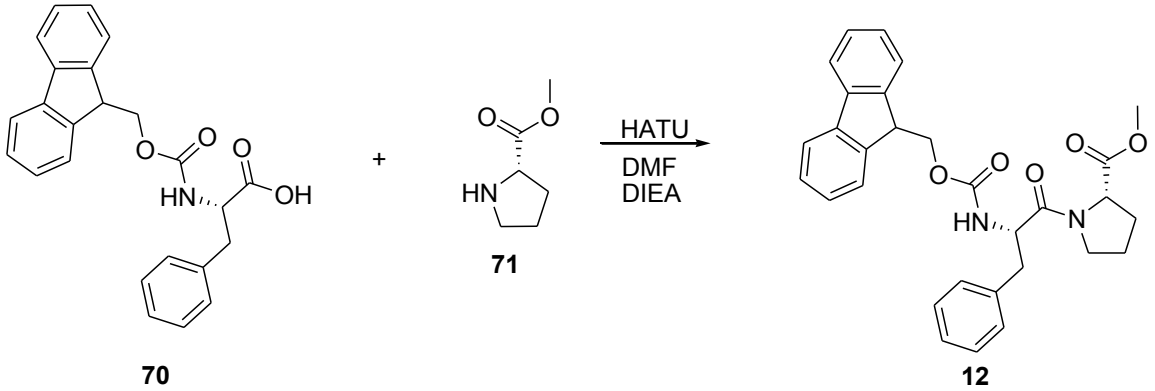
(R)-2-(benzyloxycarbonylamino)-3-(1H-indol-3-yl)propanoic acid (**16**) (Yadav, Reddy et al. 2002)



68 **16**

Scheme 1-6 Synthesis of (R)-2-(benzyloxycarbonylamino)-3-(1H-indol-3-yl)propanoic acid (**16**)

(S)-methyl 1-((S)-2-(((9H-fluoren-9-yl)methoxy)carbonylamino)-3-phenylpropanoyl)pyrrolidine-2-carboxylate (12)



70 **71** **12**

Scheme 1-7 Preparation of (*S*)-methyl 1-((*S*)-2-(((9H-fluoren-9-yl)methoxy)carbonylamino)-3-phenylpropanoyl)pyrrolidine-2-carboxylate **(12)**

((S)-2-(((9H-fluoren-9-yl)methoxy)carbonylamino)-3-phenylpropanoic acid) (387 mg, 1 mmol, 1 eq) was dissolved in DMF (10 mL). DIEA (628 μL, 3.5 mmol, 3.3 mmol) was added followed by L-proline methyl ester (182 mg, 1.1 mmol, 1.1 eq) and HATU (418 mg, 1.1 mmol, 1.1 eq) and the RM was allowed to stir overnight. The RM was concentrated in vacuo and 1.394 g of red oil was obtained which was dissolved in EtOAc (25 mL) and washed with H_2O (2x10 mL) and Brine (10 mL), dried with sodium sulfate, filtered and concentrated in vacuo to yield 1.383 mg of reddish white solid. The purified product was obtained using flash chromatography on silica using a gradient the following gradient of EtOAc:hexanes in 50 mL portions (1:9, 2:8 and 3:7) to yield 198 mg of white solid. Yield: 40%

^{1}H NMR (300 MHz, $CDCl_3$) δ 7.76 (s, 2H), 7.73 (s, 1H), 7.58 – 7.48 (m, 3H), 7.39 (t, *J* = 7.4, 3H), 7.31 (s, 3H), 7.25 – 7.17 (m, 2H), 5.77 (s, 1H), 4.73 (d, *J* = 7.6, 1H), 4.52 (d, *J* = 7.8, 2H), 4.40 – 4.29 (m, 2H), 4.26 (d, *J* = 7.0, 1H), 4.22 – 4.12 (m, 2H), 3.76 (s, 3H), 3.04 – 2.96 (m, 1H), 2.94 (s, 1H), 2.87 (s, 1H), 2.80 (t, *J* = 1.8, 1H)

.^{13}C-NMR: (75 MHz, $CDCl_3$) δ 172.22, 170.40, 155.73, 143.81, 143.79, 141.22, 136.05, 129.73, 129.72, 128.60, 128.42, 127.69, 127.67, 127.66, 127.06, 127.04, 127.02, 126.93, 125.22, 125.13, 67.04, 59.01, 53.76, 52.26, 47.07, 46.94, 46.21,38.94, 29.01, 24.89.

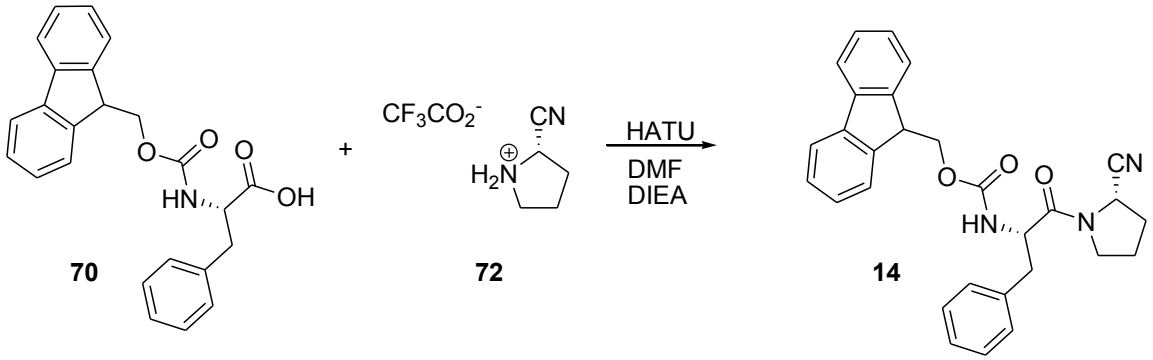
IR (Neat): 1638.3

Rf: 0.59 (2:8 hexanes/CH_2Cl_2, UV)

(S)-methyl 1-((R)-2-(((9H-fluoren-9-yl)methoxy)carbonylamino)-3-phenylpropanoyl)pyrrolidine-2-carboxylate (13)

Same procedure as for **12** using Fmoc-D-phenylalanine in place of Fmoc-L-phenylalanine

(9H-fluoren-9-yl)methyl (S)-1-((S)-2-cyanopyrrolidin-1-yl)-1-oxo-3-phenylpropan-2-ylcarbamate (14)



Scheme 1-8 Preparation of (9H-fluoren-9-yl)methyl (S)-1-((S)-2-cyanopyrrolidin-1-yl)-1-oxo-3-phenylpropan-2-ylcarbamate (**14**)

Fmoc-L-phenylalanine (217 mg, 0.56 mmol, 1 eq), DL-2-21 (129 mg, 0.61 mmol, 1.1 eq) and HATU (232 mg, 0.61 mmol, 1.1 eq) were dissolved in DMF (5 mL) then DIEA (370 μLm 2.24 mmol, 4 eq) were added and the reaction was left to stir overnight. The RM was concentrated in vacuo to yield 810 mg of yellow oil, which was dissolved in 509 mL EtOAc and washed with 1N HCl (2x10 mL, Brine (10 mL) and dried with sodium sulfate and concentrated in vacuo to yield 442 mg of yellow solid. The oil was purified on silica using a gradient of hexane 100% to hexanes:EtOAc 50:50 to yield 180 mg of yellow solid. Yield: 63%

^{1}H NMR (500 MHz, CDCl3) δ 7.49 (d, *J* = 7.5, 2H), 7.32 (t, *J*=7.6, 2H), 7.13 (t, *J* = 7.4, 2H), 7.05 (dd, *J*=4.4, 10.3 , 4H), 7.01 (t, *J* = 6.7, 3H), 5.47 (d, *J* = 8.5, 1H), 4.43 (dd, *J* = 2.7, 7.8, 1H), 4.33 (dd, *J* = 8.7, 14.7, 1H), 4.08 (m, 2H), 3.93 (t, *J* = 7.0, 1H), 3.10 (t, *J* = 7.4, 1H), 2.80 (dd, *J* = 8.6, 13.6, 1H), 2.34 (dd, *J* = 5.7, 11.7, 1H), 1.89 (s, 1H), 1.80 (ddd, *J* = 8.2, 13.0, 30.5, 2H), 1.62 (s, 1H), 1.53 (d, *J* = 10.3, 1H).

^{13}C NMR (126 MHz, $CDCl_3$) δ .170.36, 155.49, 143.46, 143.41, 135.12, 129.49, 129.33, 129.23, 129.12, 128.59, 128.40, 127.47, 127.20, 126.78, 124.85, 124.79, 119.74, 119.67, 117.27, 66.89, 53.83, 46.80, 45.95, 45.87, 39.80, 31.65, 29.45, 24.68

IR (Neat): 1714.53, 1646.01

Preparation of (9H-fluoren-9-yl)methyl (R)-1-((S)-2-cyanopyrrolidin-1-yl)-1-oxo-3-phenylpropan-2-ylcarbamate (**15)**

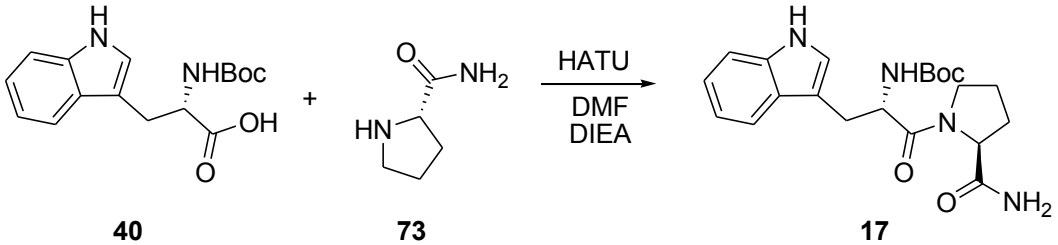
Same procedure as for **14** using Fmoc-D-phenylalanine in place of Fmoc-L-phenylalanine

^{1}H NMR (500 MHz, cdcl3) δ 7.48 (d, *J* = 7.5, 2H), 7.32 (t, *J* = 7.6, 2H), 7.12 (t, *J* = 7.3, 2H), 7.05 (dd, *J* = 4.4, 10.3, 4H), 6.98 (t, *J* = 7.0, 3H), 5.48 (d, *J* = 8.7, 1H), 4.39 (dd, *J* = 9.2, 14.6, 1H), 4.25 (dd, *J* = 6.1, 1H), 4.08 (m, 2H), 3.93 (t, *J* = 6.9, 1H), 3.26 (t, *J* = 7.4, 1H), 2.82 (dd, *J* = 5.4, 12.8, 1H), 2.75 – 2.67 (m, 1H), 2.25 (dd, *J* = 9.2, 16.2, 1H), 1.83 (s, 1H), 1.72 (s, 1H), 1.52 (d, *J* = 10.3, 1H), 1.42 (s, 1H),

^{13}C NMR (126 MHz, $CDCl_3$) δ 170.19, 155.32, 143.43, 143.37, 140.99, 135.61, 129.19, 129.11, 128.42, 128.39, 128.28, 127.45, 127.45, 127.30, 126.88, 124.70, 119.82, 119.69, 117.62, 66.92, 53.88, 46.83, 46.07, 45.87, 39.79, 31.97, 29.54, 24.38

IR (Neat): 1713.55, 1651.72

tert-butyl (S)-1-((S)-2-carbamoylpyrrolidin-1-yl)-3-(1H-indol-3-yl)-1-oxopropan-2-ylcarbamate (17)



40 **73** **17**

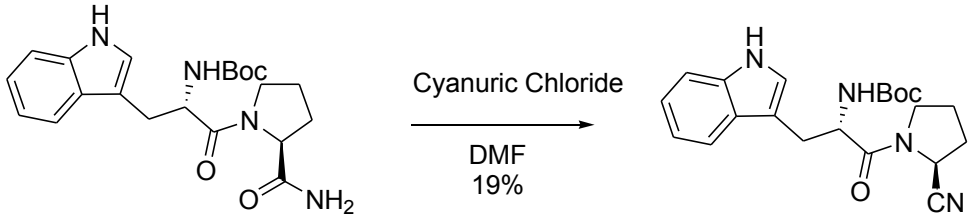
Scheme 1-9 Preparation of tert-butyl (S)-1-((S)-2-carbamoylpyrrolidin-1-yl)-3-(1H-indol-3-yl)-1-oxopropan-2-ylcarbamate (**17**)

((S)-2-(tert-butoxycarbonylamino)-3-(1H-indol-3-yl)propanoic acid) (700 mg, 1.73 mmol, 1 eq), L-proline amide (217 mg, 1.90 mmol, 1.1 eq) HATU (724 mg, 1.90 mmol, 1.1 eq) and DIEA (943 μL, 5.71 mmol, 3.3 eq) were added to a round bottom flask under argon and left to stir for 24 hours. The RM was concentrated in vacuo to yield 3.392 mg of yellow/orange solid. The crude mixture was dissolved in EtOAc (60 mL). The organic phase was washed with H_2O (2x15 mL) and HCl (0.12 M, 10 mL), brine (10 mL) and dried with sodium sulfate, filtered and concentrated in vacuo to yield 1.05 g of yellow/white crystals. 25 mg of the sample was purified via HPLC at the concentration of 100 mg/ mL with a Zordax preparatory column. Yield: 1%

^{1}H NMR (200 MHz, DMSO) δ 10.85 (s, 1H), 7.56 (d, *J* = 7.3, 1H), 7.32 (d, *J* = 7.9, 1H), 7.21 (s, 2H), 7.02 (dd, *J* = 7.1, 15.2, 2H), 6.92 (s, 2H), 4.39 (s, 1H), 4.25 (s, 1H), 3.60 (s, 2H), 3.09 (d, *J* = 14.6, 1H), 2.89 (d, *J* = 9.3, 1H), 1.83 (s, 3H), 1.31 (d, *J* = 11.5, 9H), 1.11 (d, *J* = 2.1, 2H).

Rf: 0.52 (9:1 EtOAc:MeOH, UV)

tert-butyl (S)-1-((S)-2-cyanopyrrolidin-1-yl)-3-(1H-indol-3-yl)-1-oxopropan-2-ylcarbamate (18)



17 **18**

Scheme 1-10 Preparation of tert-butyl (S)-1-((S)-2-cyanopyrrolidin-1-yl)-3-(1H-indol-3-yl)-1-oxopropan-2-ylcarbamate (**18**)

(tert-butyl (S)-1-((S)-2-carbamoylpyrrolidin-1-yl)-3-(1H-indol-3-yl)-1-oxopropan-2-ylcarbamate), (600 mg, 1.49 mmol, 1 eq) was chilled to around 0°C with cyanuric chloride (179 mg, 0.974 mmol, 0.65 eq) in DMF (2 mL). The RM was left to stir overnight under argon. After 20 hours of reaction time, TLC showed that SM was still present. Cyanuric chloride (10 mg) was added and the RM was left to stir for another 24 hours. The RM was quenched with H_2O (10 mL), extracted with EtOAc (3x10 mL), the extract was washed with LiCl (10%, w/w, 5 mL), dried with sodium sulfate and filtered. After concentration in vacuo 697 mg of brown oil. The crude mixture was purified by flash chromatography using silica with ~1 mL of Et_3N added to the silica slurry. The gradient used was: hexanes:EtOAc 100:0, 40:60, 60:40, followed by MeOH:EtOAc 20:80 mL. 94 mg of white solid was obtained. Yield 19%

^{1}H NMR (500 MHz, $CDCl_3$) δ 7.37 (s, 1H), 6.87 (d, J = 7.6, 1H), 6.52 (d, J = 8.0, 1H), 6.42 (d, J = 1.7, 1H), 6.33 (dt, J = 7.2, 15.2, 2H), 6.26 (s, 1H), 4.49 (d, J = 7.7, 1H), 3.84 (s, 2H), 2.47 (d, J = 8.0, 1H), 2.42 (d, J = 13.7, 1H), 2.36 – 2.28 (m, 1H), 1.77 (s, 1H), 1.18 (d, J = 17.8, 2H), 0.94 (s, 1H), 0.62 (d, J = 18.5, 9H)

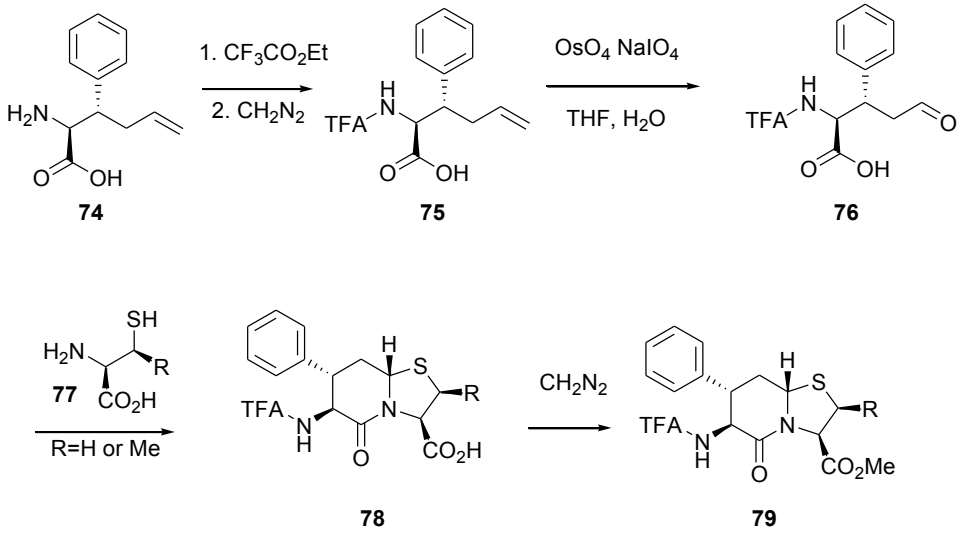
^{13}C NMR (75 MHz, $CDCl_3$) δ 171.71, 155.24, 136.04, 127.35, 124.02, 122.11, 119.77, 118.41, 118.15, 111.34, 109.17, 79.93, 52.38, 46.03, 45.97, 29.62, 29.45, 28.37, 24.86.

Rf: 0.66 EtOAc/Hexanes 7:3, vanillin stain

Chapter 2: Bicyclic scaffolds

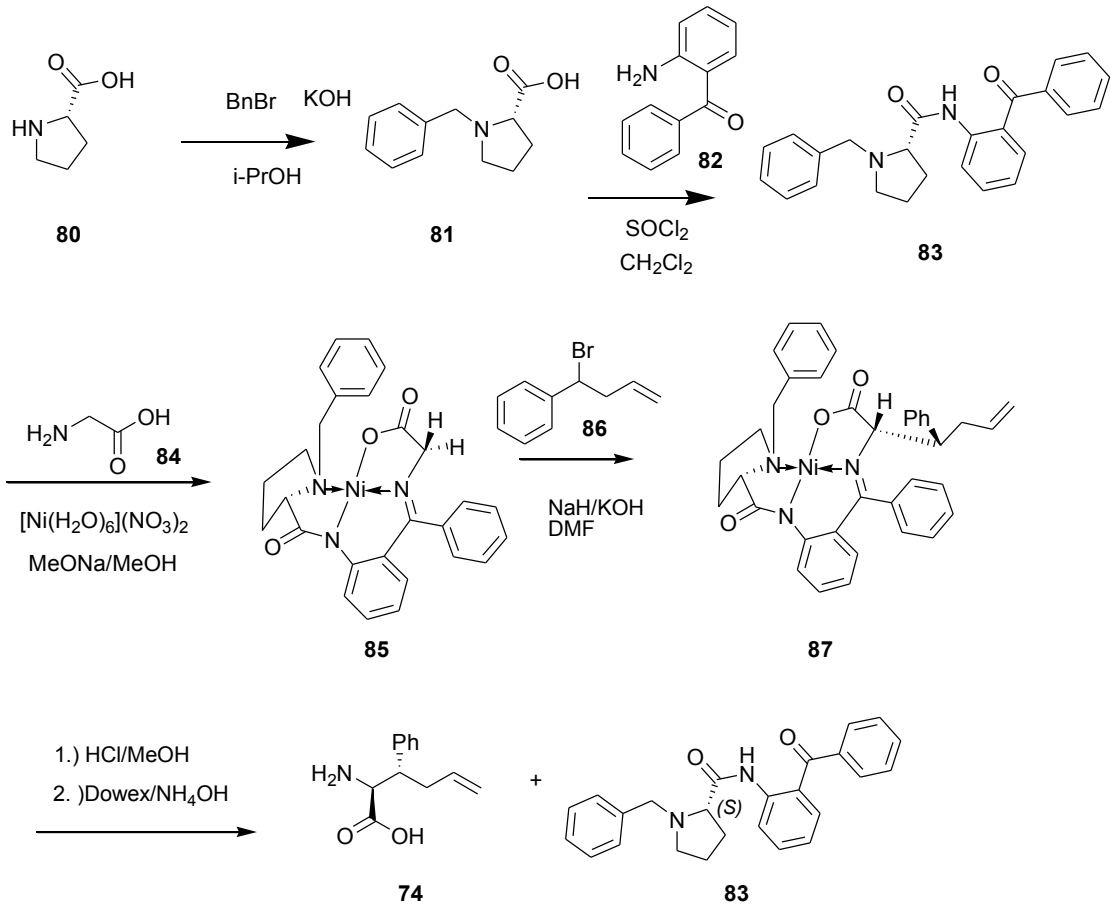
2.1 Introduction

In order to explore the effects of rigidifying similar potential inhibitors based on the previously described dipeptide inhibitors, the synthesis of bicyclic compounds was started. The general route is described in Scheme 2-1 . The starting material shown in Scheme 2-1 is functionalized allyl amino acid and was needed in order to provide the intermediate that was able to cyclize with cysteine, providing the bicyclic compound (Gu, Cowell et al. 2003). Compound **79** was chosen as a target because of bicyclic β-turn dipeptides may yield more active compounds than their non-rigidified analogues. For example, if the methyl ester group were transformed into a nitrile group, this would mimic the proline nitrile in compound **14**.



Scheme 2-1 Synthesis of bicyclic scaffold from functionalized allyl amino acid

In order to form the desired a functionalized allyl amino acid, several steps involving the formation of a nickel complex were completed in order to selectively alkylate the glycine building block (Scheme 2-2).



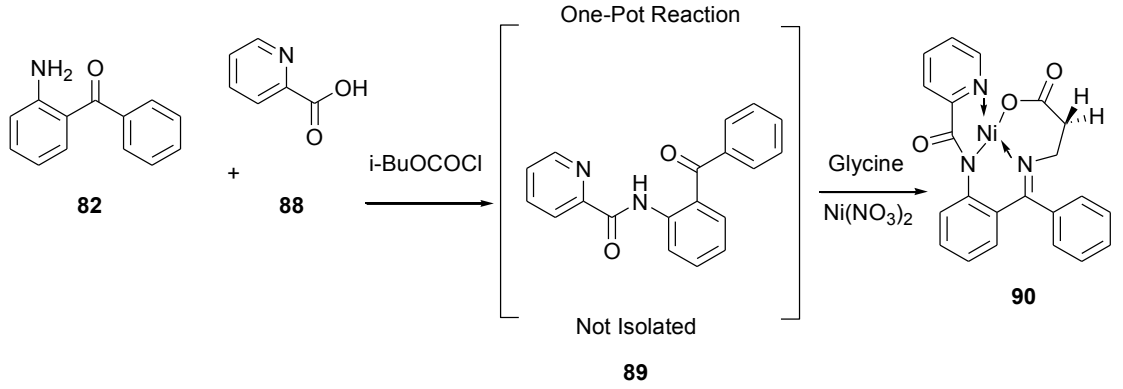
Scheme 2-2 Overall Scheme to provide desired functionalized allyl amino acid

2.2 Formation of the Nickel Complex

Using the original procedure by Belokon (Belokon, Tararov et al. 1998) to form the nickel complex proved to be very difficult. The attempted coupling of (*S*)-2-[*N*-(*N*′-benzylprolyl)amino]benzophenone (BPB) and glycine using a variety of bases such as KOH (Belokon, Tararov et al. 1998) and MeONa/MeOH (Popkov, Gee et al. 2002) did not result in coupling. Dry glassware, inert atmosphere were used in all of the initial coupling attempts but did not produce the desired material Therefore the lack of reproducibility had to be addressed.

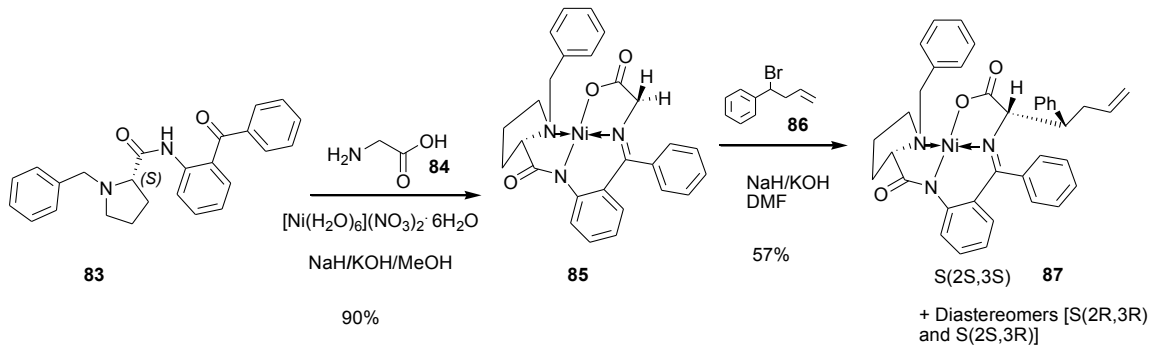
The method which used NaH to remove the excess water was obtained by modifying a similar reaction published by Deng et al. (Deng, Wang et al. 2007) in which a similar nickel complex, (2-[N-(a-picolyl-amino]benzophenone) was made in good yield in

seemed unaffected by ambient moisture. Scheme 2-3 shows the one pot reaction which circumvents the need for forming the benzyl proline by substituting the commercially available picolinic acid.



Scheme 2-3 A one pot reaction for (2-[N-(a-picolyl-amino]benzophenone)

The problem with the formation of the nickel complex with glycine and nickel nitrate was identified as the presence of water in the reaction mixture. The aforementioned procedure (Scheme 3-2) used to form the nickel complex was changed so that excess water was removed *in-situ*. The nickel complex was obtained by adding sodium hydride to form a base mixture that allowed controlling the excess water in the reaction (Scheme 2-4). The desired product was obtained in high yield and the first batch of crystals appeared to be very clean, therefore no chromatography was needed as described in the original method described by Belokon (Belokon, Tararov et al. 1998). Further material was obtained from the filtrate that was less pure, and was easily purified. The spectra showed identical peaks as described in the literature (Ueki, Ellis et al. 2003).



Scheme 2-4 Formation of Alkylated Nickel Complex

The nickel complex was then alkylated with the previously prepared 1-bromo-3-butenyl-benzene (**86**) using 3 equivalents of the allyl bromide which favours the production of the *S*(*2S,3S*) diastereomer over the *S*(*2R,3R*) and S(*2S,3R*) diastereomers. The reaction was first carried out using 0.474 mmol (236 mg) of the nickel complex and a mixture of compounds was obtained. The two major spots by TLC were isolated using flash chromatography and the two major fractions were obtained in 26% and 31% yield. However, neither fraction produced a ^{1}H-NMR spectra which exactly matched the literature values (Popkov, Nadvornik et al. 2008). This was expected, since the literature values were obtained from a highly purified sample which was obtained for reference purposes, but a less pure product was used for the synthesis. The authors state that the *S*(*2S,2R*) coexists as a minor product and which is extremely difficult to separate. The key intermediate **74** is currently being prepared in sufficient quantity for the cyclization with cysteine as described in Scheme 2-1.

2.3 Discussion

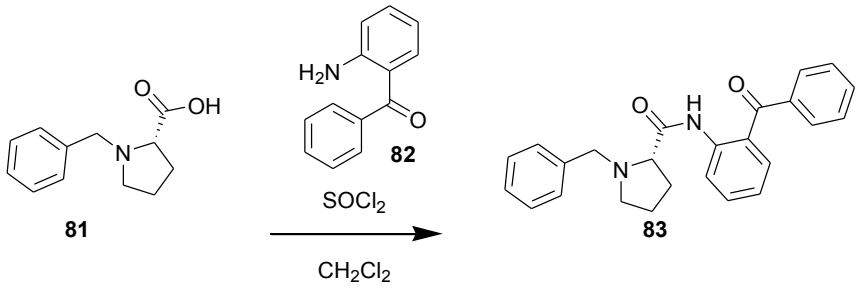
The synthesis described in Scheme 2-1 is still in progress and should be completed in the coming month. The bicyclic system that was being investigated would have provided an interesting scaffold in which further elaboration using the knowledge gained from the SAR of the dipeptide inhibitors of DPP-IV could have been used. Further work includes the reproduction of Belokon's original work followed by elaboration of the bicyclic scaffold to explore the what effects the functional groups such as tetrazoles and nitriles would have on DPP-IV inhibition.

2.4 Conclusion

The effects of rigidified version of active dipeptides can potentially add to the activities of the compounds due to the entropic advantages if the rigidified compound has a similar conformation of the active linear dipeptides. Modeling studies based on previous work in the group (Lawandi, Toumieux et al. 2009) suggests that an increase in activity will be obtained by introducing rigid scaffolds.

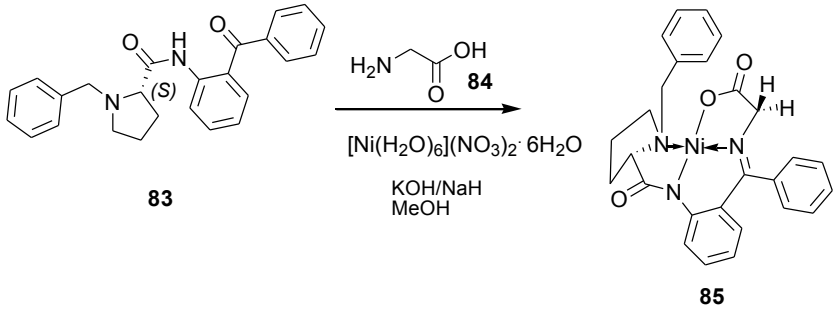
2.5 Experimental Section

Benzylprolyl-(*S*)-2-[*N*-(*N′*-benzylprolyl)amino]benzophenone 83 (BPB) by Belokon (Belokon, Tararov et al. 1998)



Scheme 2-5. Formation of Benzylprolyl-(*S*)-2-[*N*-(*N′*-benzylprolyl)amino]benzophenone **85** (BPB)

Nickel glycine complex 85



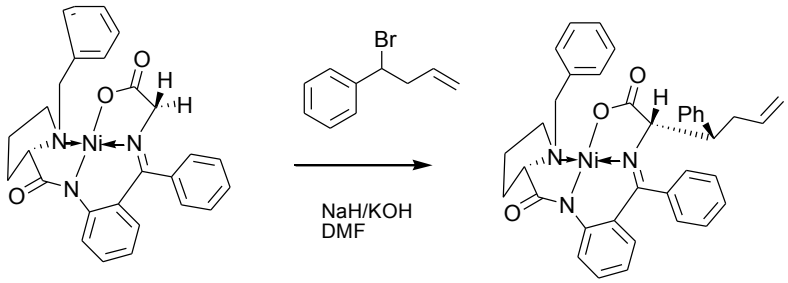
Scheme 2-6 Modified procedure for formation of **85**

Procedure for the formation of nickel glycine complex 85 (Based on a combination of Belokon's (Belokon, Tararov et al. 1998) **procedure and Deng's procedure** (Deng, Wang et al. 2007))

BPB (1.308 g, 3.4 mmol, 1 eq) was dissolved in MeOH (20 mL) and glycine (1.275 g, 17 mmol, 5 eq), $Ni(NO)_3.6H_2O$ (1.977 g, 6.8 mmol, 2 eq), NaH (60%, 815 mg, 19.5 mmol, 5.8 eq) and KOH (572 mg, 10.2 mmol, 3 eq) were added and the RM was refluxed for 2 hours. The reaction was cooled and neutralized to pH 6 with dilute HCl and left to stir overnight. 2.8 g of red solid was filtered off and washed with ethanol (30 mL) After drying the solid in vacuo 998 mg of red solid was obtained. Yield: 59%

^{1}H NMR (300 MHz, $CDCl_3$) δ 8.27 (d, *J* = 8.1, 1H), 8.06 (d, *J* = 6.9, 2H), 7.54-7.48 (m, 3H), 7.42 (t, *J* = 7.5, 2H), 7.33-7.17 (m, 2H), 7.09 (m, 2H), 6.97 (m, 1H), 6.79 (m, 1H), 6.70 (m, 1H), 4.09 (d, *J* = 12.6, 2H), 3.75 – 3.64 (m, 3H), 3.47 (dd, *J* = 5.7, 1H), 3.35 (m, 1H), 2.56 (m, 1H), 2.44 (m, 1H), 2.19 – 2.06 (m, 2H)

Alkylated nickel complex 86 (Belokon, Tararov et al. 1998)



85 **86**

Scheme 2-7 Alkylation of **85**

Chapter 3: Virtual Screening[1]

Abstract

The use of predictive computational methods in the drug discovery process is in a state of continual growth. In recent years, an increasingly large number of docking tools used for the identification of leads or hits by docking chemical libraries to proteins *in-silico* have been developed. In this study, the impact of protein flexibility and water molecules on the accuracy of eight docking programs (eHiTS, FITTED, FlexX, Glide, GOLD, Surflex, LeadFinder and PLANTS) to discriminate active compounds from inactive compounds in VS campaigns was determined. A total of 27 co-crystallized proteins representing seven unique enzymes and receptors and sets of 1000 known ligands and decoys were selected from the PDB and the DUD respectively.

3.1 Introduction

Traditional medicinal chemistry has been instrumental to develop a number of drugs. However, for the last thirty years or so, constant advances in robotics, spectroscopy and computer science and hardware have introduced novel strategies for drug design. High throughput screening (HTS), fragment-based drug design and computational drug design techniques are among the most popular approaches currently used. At the hit discovery stage, HTS and its *in-silico* counterpart virtual screening (VS) are well established methods (Rose and Stevens 2003). The combination of advancements in knowledge, CPU power and an increase in speed at which chemical compounds can be made and tested against activity for a given biological target has led us to a point where computational methods have become a recognized and respected tool for drug design.

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[1] In preparation to be submitted to the Journal of Chemical Information and Modeling:
Docking Ligands into Flexible and Solvated Macromolecules. 6. Impact of Protein Flexibility and Water Molecules on Docking-based Virtual Screening Accuracy
Devin Lee, Eric Therrien, Christopher R. Corbeil and Nicolas Moitessier*

The number of available proteins that can be used for 3D structure based VS is currently growing at a tremendous rate. With over 60000 protein structures housed at the Research Collaboratory for Structural Biology (RCSB) Protein Data Bank (PDB) which itself is growing with thousands of proteins being added each year.

Virtual screening (VS) of medium to large libraries of compounds has become a routine practice as a method to quickly increase hit rates in lead discovery. More specifically, structure-based VS generally involves docking of a library of small molecules to a protein structure followed by a rank ordering of these molecules by predicted binding affinities. In practice, proper docking of a ligand into a protein *in-silico* depends on a wide variety of factors, such as an exhaustive conformational search of the ligand and proper identification and use of interactions of the ligand and the residues (and metals where applicable) within the binding site (Corbeil and Moitessier 2009). For the second step, scoring functions have been developed to predict binding affinities and/or to place the most likely active compounds at the top of the list (Englebienne and Moitessier 2009). The combination of highly accurate docking programs and scoring functions is expected to significantly impact drug discovery programs.

In order to achieve optimal results from a structure-based VS involving protein-ligand docking, several factors need to be considered. First, the protein structure to be studied must be obtained with a reasonable resolution and if possible bound to a small molecule, as unbound protein structures tend to give less reliable results(Kitchen, Decornez et al. 2004). In fact, ligand-bound proteins often adopt a conformation different from the apo form. In addition, the protein conformation is also often dependent on the ligand bound.

When undertaking a lead discovery program, choosing between two methods (or a combination thereof) such as HTS or VS is of great importance as the associated costs, required time and personnel are very different. Screening compounds *in-silico* offers a number of benefits. A VS study is easier to set up, faster to run, cheaper and more environmentally friendly. As the processing power available to chemists increase and docking/scoring methods are fine-tuned, the role in fast and accurate VS will be play an increasingly important role in drug design. But just as important as the aforementioned choice, is the choice of docking program(s) to use if the VS option is chosen for lead

identification. At this stage, both knowledge of the protein targets and knowledge of the docking tools used to study them are of paramount importance. Certain proteins are more flexible than others, and in such cases, it stands to reason that a program that handles protein flexibility reliably should be used over a program that does not consider protein flexibility. Handling of conserved waters is another factor that needs to be addressed in a similar fashion. Deciding which water molecules are important in binding requires knowledge of the protein. And as the results of this study show, the use of conserved waters in the protein during docking can have a significant effect on the results, depending upon the program's ability to handle waters.

It is unlikely that one particular piece of software (often trained on a specific and limited set of proteins) is universally superior, thus validation of a specific piece of software to each unique VS should be done. While a study such as this one can show the strengths and weaknesses of a certain program, ultimately the effectiveness of a VS will depend on many factors unique to a particular VS. In fact, even though we foresee that this report will be received as a comparative study of docking programs, our intention is to complement our previous two studies on the effect of water molecules, protein hydropathicity and protein flexibility on: 1. pose prediction (Corbeil and Moitessier 2009) and 2. Scoring function accuracy (Englebienne and Moitessier 2009) by the study of their impact on docking-based VS campaign success rate. A number of comparative studies have been reported to highlight strengths and weaknesses of the most popular programs (Moitessier, Englebienne et al. 2008). These reports were followed by a number of reports describing the pitfalls of these studies, their fairness, statistical significance and transferability to other proteins. We have been developing FITTED, a docking tool with a major focus on protein flexibility and water molecules. Expectedly, implementing protein flexibility and water molecules were found to improve its accuracy. Our first validation study of docking programs for their ability to predict the binding mode of ligand has shown that most of the major docking programs are affected by protein flexibility and to less extent to water molecules (Corbeil, Englebienne et al. 2007). We have also looked at the biases such as input ligand conformation and attempted to compare the program reducing these biases (Corbeil and Moitessier 2009).

In this study eight docking programs (eHiTS, FITTED, FlexX, Glide, GOLD, LeadFinder, PLANTS and Surflex) were applied to specifically selected proteins. The key focal points of this study included: the effects of protein flexibility, the effects of conserved water molecules in the binding site and the transferability to various proteins. The results and conclusions from this study will provide valuable information for the end users of docking tools as well as valuable data for the developers of these tools for future improvements and refinements.

It is hoped that this study will further the general knowledge base in docking-based VS and help future and current users of VS tools to decide what tool is most appropriate to use for any given target and or application. In order to provide information that can be used for future comparisons, all data collected will be published in a raw format, which should lend itself to further examination. As noted by Sheridan *et al*.(Sheridan, Singh et al. 2001), since VS results are so dependent on the exact details of the query and the content of the database, it is nearly impossible to compare methods between different VS studies when none of these parameters are in common, and only enrichment metrics are published.

3.2 Methods

3.2.1 Actives and decoys

It is useful to start by clarifying some definitions that have a specific meaning in VS studies but may have different meanings in another context. In a typical VS study, there are potentially a certain number of active compounds in a large library and the goal is to use the VS method to identify these actives. The accuracy of a VS tool is evaluated using a set of actives combined with a second set of inactives. An active compound is one that is often known to be active through biological assays, however, the inactives, which will be referred to as decoys from this point forward are presumed to be inactive. While it is possible to prove that all decoys are in fact not active, this is not really feasible, especially when considering the screening of databases that include over a million compounds.

3.2.2 Ligand datasets and protein structures

While the docking tool should be able to reproduce known binding modes, the true value of conducting a VS is its ability to find unique ligands with potentially novel binding modes.(Ewing, Makino et al. 2001) Thus the performance of docking tool is typically assessed by observing its ability to reproduce experimentally observed binding modes. In the context of VS, the accuracy can be assessed by virtually screening a set of ligands containing a small number of known actives with a large number of inactives (decoys) to rank order the actives over the inactives. For VS, metrics such as the enrichment factor (EF) or the receiver operating characteristic ROC curves can be used to measure success (Shoichet 2004).

Both of these methods of validation suffer from shortcomings such as being susceptible to the ratio of actives to decoys in the case of EF and a less than perfect ability to recognize early enrichment for ROC. Ideally, the decoys should resemble the active ligands in physical properties so that the enrichment is not simply a separation of obvious physical features (i.e., molecular size, charge), but at the same time they must also be chemically distinct from the actives to assure that they are inactive (non-binders). A number of sets of actives and decoys have been reported but do not address this issue. The directory of useful decoys (DUD) (Huang, Shoichet et al. 2006) was made to address this issue and reduce this bias. The DUD is a database composed of 1950 ligands for 40 different targets in which each ligand has 36 corresponding decoy molecules that were chosen to be physically similar while being topologically distinct. Thus, this set is a suitably challenging set for evaluating the ability for the VS tools studied herein to rank order active compounds over inactive compounds (actives over decoys).

Initial investigation using the DUD benchmark involved an exhaustive search for true duplicates in order to be certain that the true duplicates were removed prior to the analysis of the final docking study. Each of the compounds that composed the subset derived from the DUD set in this study was unique in structure. While the Zinc IDs of the compounds may be identical, these instances represent tautomers of the identical compounds. A script was then written that pruned the original DUD list so that each

protein ligand set would contain 40 original actives plus tautomers and 960 decoys plus tautomers. In the cases of TK and ER, less than 40 actives were available in the DUD set.

Seven representative enzymes (CDK2, ER, HIVP, HIVRt, P38, TK, Thrombin) from four different protein families (serine and aspartic proteases, nuclear receptors, reverse transcriptases and kinases) were selected. This set of protein structures was chosen based on resolution and other factors such as the presence or absence of "conserved waters". A water molecule was defined to be a "conserved water" if the water molecule was observed in similar position within the active site in multiple crystal structures of the same protein. Unlike the original DUD docking study which introduced the DUD to the scientific community, in which a single X-ray crystal structure was used, a number of crystal structures were obtained from the PDB for any given enzyme. The number of unique crystal structures used for a given enzyme was dependent of the availability and quality of the crystal structures posted on the PDB database. Keeping an emphasis on the chemical diversity of the ligand (when possible), the number of unique crystal structures finally varied from three to four structures. Including more than one X-ray crystal structure for each enzyme was a key element for the determination of the effect of receptor flexibility.

Protein	Target Type	PDB ID	Actives	Decoys
CDK2	Kinase	1aq1, 1dm2, 1pxn, 1pxp	57	1173
ER	Nuclear Hormone Receptor	1err, 1sjo, 3ert	39	1033
HivPr	Protease	1pro, 1b6l, 1hpv, 1hpo	48	1098
HivRt	Reverse transcriptase	1c1b, 1rt1, 1vrt, 1fk9	43	1049
P38	Kinase	1w7h, 1w82, 1w84, 1a9u	66	1111
Thrombin	Protease	1dwc, 1etr, 1tmt, 1ett	41	1074
TK	Kinase	1e2k, 1of1, 1ki3, 2ki5	22	913

Table 3-1 Selected protein structures and corresponding PDB codes

Cyclin-Dependent Kinase 2 (CDK2). CDK2 is a member of a large family of protein kinases that initiates the principal transitions of the eukaryotic cell cycle and is an important drug target for cancer therapy. CDK2 is of particular interest since X-ray crystallographic studies have shown that two potential binding modes exist in CDK2 (Sato, Shewchuk et al. 2006). Conserved waters are observed in CDK2. In each case studied, one conserved water is observed, except for the case of 1dm2, which has two waters which were deemed significant and so were kept during the study. It has already been reported by Thomas et al. (Thomas, McInnes et al. 2005), that in docking studies the binding is affected by the amount that the Lys33, Phe80, Lys89 and Asp145 protrude into the ATP binding side. Kim *et al.* (Kim, Park et al. 2007) showed by superimposing 17 CDK-2 proteins that the backbone conformation varies very little, but that there are significant differences in the side chain conformation of the four amino acid resides previously mentioned. Figure 3.1 shows the native ligands from the CDK-2 proteins used for the study.

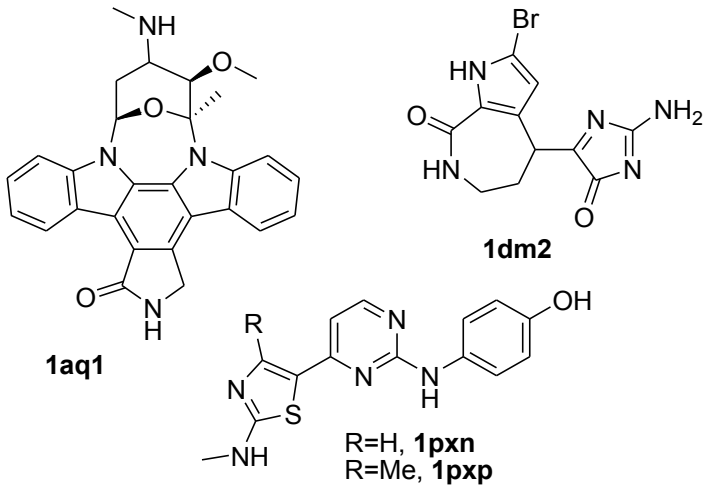

Figure 3.1 Native ligands from crystallized CDK-2 proteins

Estrogen Receptor Antagonists (ER). ER has a deeply buried hydrophobic binding site and its binding cavity is therefore less open to solvent than that of other targets such as TK (Bissantz, Folkers et al. 2000). In addition, the topology of the active site shows less dependency on the nature of the ligand when bound to the active site. The receptor is a large steroid-sized lipophilic cavity possessing acceptor groups at either end which

provide possible sites for hydrogen bonds to be formed with properly positioned hydroxyl groups on the ligand. Lipophilic interaction energies are of great importance for both ER agonists and antagonists. In this study we are studying the antagonists which is an important difference to take note of since most antagonists form an additional salt bridge to Glu351 (Stahl and Rarey 2001). The importance of this salt bridge is expected to show some effect in the VS results when comparing the docking to the "wet proteins" compared to the "dry proteins and "H_2O proteins", which are the terms used to define the existence of the crystallographic waters and how the waters are used by the docking programs. The finer details of these runmodes will be discussed in the methods section. The actual effects of salt bridge will be examined in the results and discussion section.

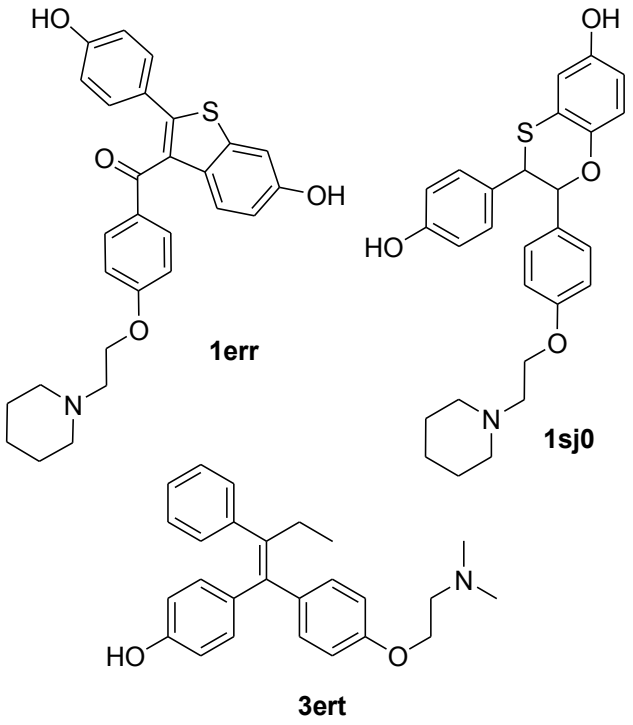

Figure 3.2 Native ligands from ER antagonists

HIV-1 Protease (HIVP). HIVP is an aspartic protease responsible for cleaving newly synthesized polyproteins to create the mature protein components of an infectious HIV virion. HIVP is an important drug target since its inhibition disrupts HIV's ability to

replicate and infect additional cells(Furfine, D'Souza et al. 1992). The protein exists as a homodimer with the active site existing between identical subunits and has the characteristic Aps-Thr-Gly sequence.

The enzyme binds relatively hydrophobic peptides with multiple inter-molecular hydrophobic substituents. The binding site in HIV-1 protease is buried and is mostly hydrophobic. The shape of the binding site is oblong, which makes large and flexible ligands suitable binders. Due to the restrictive size and shape of this binding site, this target was expected to be a challenge for VS. A conserved catalytic water molecule is also an integral part of the binding site. This provides an interesting test for the VS tools examined and their ability to dock and store interactions with water molecules (Martin, Begun et al. 1999).

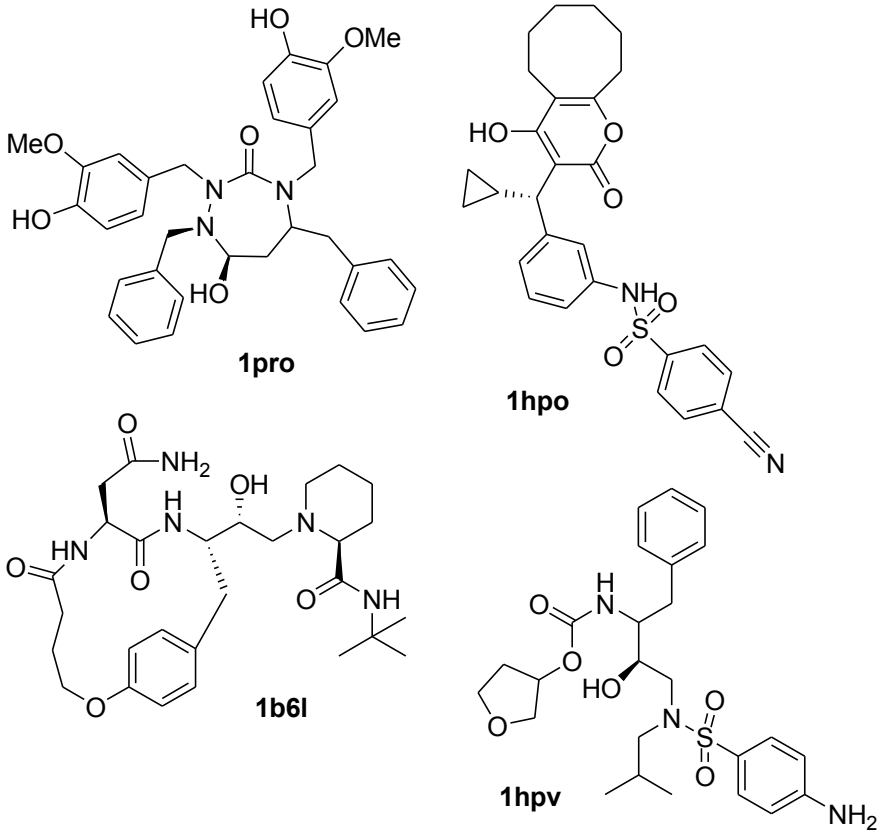

Figure 3.3 Native ligands from HIVP proteins

HIV Reverse Transcriptase (HIVRt). The reverse transcriptase (RT) enzyme (also known as RNA-dependent DNA polymerase) which transcribes single stranded RNA into double stranded DNA. HIV uses RT to copy its genetic material and generate new viruses as part of the proliferation cycle of the retrovirus. A number of drugs inhibiting this enzyme have already been developed.

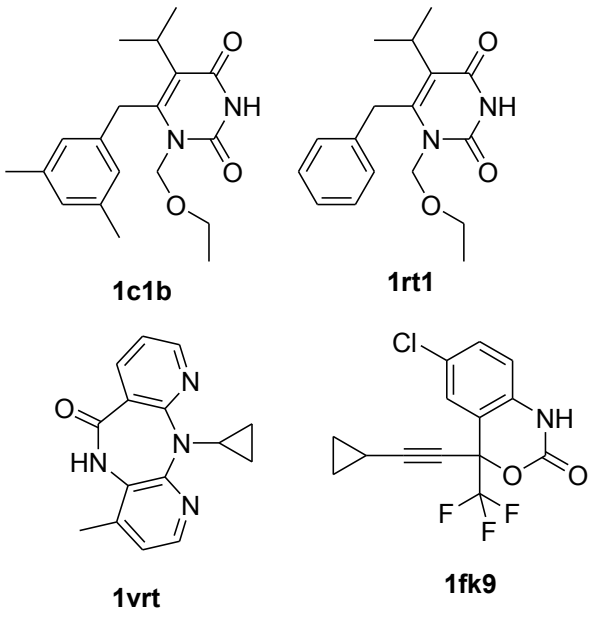

Figure 3.4 Native ligands from HIVRt proteins

Mitogen-activated protein kinases (P38). P38 MAP kinases are a class of mitogen-activated kinases which are involved in cell differentiation and apoptosis. This class of protein kinases respond to stress stimuli such as heat or osmotic shock, UV irradiation and cytokines. The binding site of P38 is mostly hydrophobic and relatively buried. These features and the absence of narrow subpockets suggest that P38 is a relatively easy target for docking.

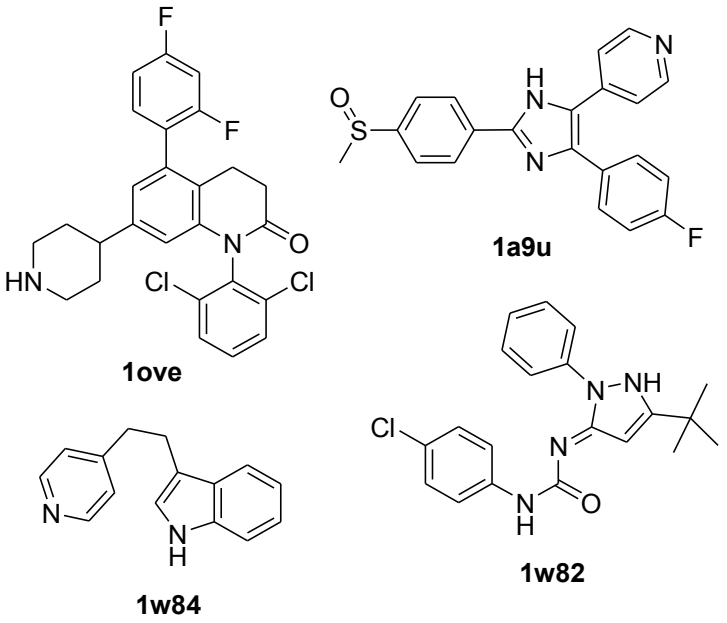

Figure 3.5 Native ligands from P38 proteins

Thrombin (THR). Thrombin (activated Factor II [IIa]) is a coagulation protein. As a serine protein that converts soluble fibrinogen into insoluble fibrin, it has many effects in the coagulation cascade. The activation of prothrombin is crucial for coagulation and it has been suggested that anti-thrombin antibodies in autoimmune disease may play a role in the formation of lupus anticoagulant.

As expected for a protease there exist a number of important hydrogen bonds to be considered in the binding of a ligand to the active side. In addition to this, the active site includes a subsite that binds basic groups (S1) and a subsite (S2) that binds an aromatic group (S4).

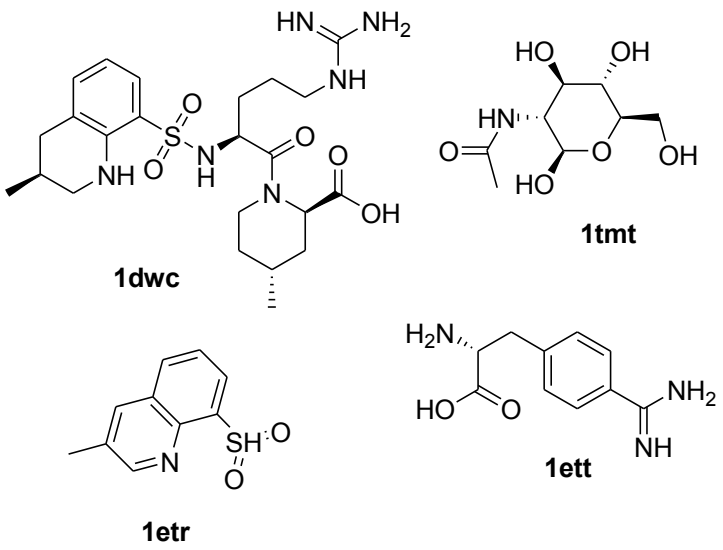

Figure 3.6 Native ligands from Thrombin proteins

Thymidine Kinase (TK). TK presents a challenging target for a VS because its binding cavity is easily accessible to water and the rotameric states of the side chains within the active site are easily influenced by induced fit.(Bissantz, Folkers et al. 2000) It is expected that VS tools with algorithms that consider protein flexibility to have an advantage over VS tools that do not consider protein flexibility in the case of TK. Another complicating factor is due to the participation of water molecules which differs depending on the chemical series to which the ligands belong. The two main series are purines and pyrimidines-like substrates which can be seen in Figure 3.6

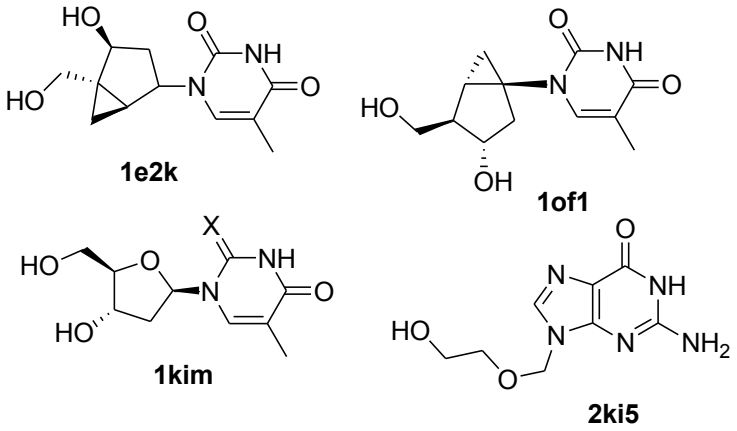

Figure 3.7 Native ligands from Thymidine Kinase proteins

When multiple receptor conformations are not considered during a VS, potentially active ligands may be misranked due to clashes with the receptor, or due to incorrectly calculated ligand-protein contacts which are not strong enough to hold the ligand in the correct position. Also, while the docking may be correct, the scoring may be incorrect for a variety of reasons which go beyond the scope of this research.

The comparison of speed is a surprisingly complex topic due to the many factors that need to be considered. For example, if one program needs to be run twice, once dry and once wet in order to consider the impact of the water molecule, while another program handles the water molecules in one run. Another example would be the comparison of one program that handles protein flexibility by docking to multiple enzymes and making a conformational ensemble, compared to another program that can use a single crystal structure to take into account protein flexibility.

3.3 Selected Docking Tools

Eight VS tools were chosen for the comparative study based on the availability of the software as well as their performance based on previous comparative studies (Maria, Glenn et al. 2005), (Friesner, Banks et al. 2004). The VS tools that were selected included GLIDE (Schrödinger),(Friesner, Banks et al. 2004; Friesner, Murphy et al. 2006) GOLD

(Marcel, Jason et al. 2003), SurFlex-Dock (UCSF, Tripos (Jain 2003; Jain 2007) eHiTS (SimBioSys Inc) (Zsoldos, Reid et al. 2007) FITTED (CORBEIL, ENGLEBIENNE ET AL. 2007; CORBEIL AND MOITESSIER 2009) FlexX (BioSolveIt) (Cross 2005) LeadFinder (Stroganov, Novikov et al. 2008) and PLANTS (Korb, Stutzle et al. 2006).Although these programs have been described elsewhere, a brief description is given below with a focus on protein flexibility and water molecules. For a review of docking programs, scoring functions and algorithms implemented in docking programs see:the review by Moitessier et al. (Moitessier, Englebienne et al. 2008).

In order to achieve success in a VS study, one must have the expertise of using the tools at hand, and also be knowledgeable about the target that is being studied. For this reason, whenever possible, expert opinion from the developers was used in order to ensure the quality of the results.

eHiTS 2009 (electronic High Throughput Screening). eHiTS uses a systematic docking algorithm with a divide and conquer approach to the docking problem. The ligand is divided into rigid fragments with flexible connecting chains, and the fragments are docked independently into the receptor site at which point another algorithm finds the poses of each fragment to re-form the input ligand. Thanks to this systematic algorithm with no random, stochastic or evolutionary elements, a comprehensive search space is covered. All major docking modes compatible with steric and chemical constraints of the target cavity are generated, outputting multiple sets of 3D coordinates per structure with rough fitness scores. These rough solutions are then used to predict more exact binding modes and affinities.

Protein flexibility is partially handled by eHiTS by providing a “soft representation” of the receptor (soft quadratic potential). The scoring function uses temperature factor information contained in the PDB file in order to give a more comprehensive picture of potential interactions. The probability of atom positions is used to create a derived empirical scoring function. The -OH groups of the Ser, Thr and Tyr residues and the $-NH_3^+$- group of the Lys are rotatable, however no heavy atoms of the side or main chain

can moved in this process. Finally, the van der Waals potential is considered with a softer quadratic potential instead of a harder potential used in other force fields.

The developers recommend removing any water molecules prior to docking and no features are implemented to account for water molecules in a displaceable manner.

FlexX 3.0. FlexX employs an incremental reconstruction algorithm in which base fragments are first identified followed by placing the selected fragments into the active site of the receptor using a hashing technique. The remaining components are added to the base fragment in a step by step manner to complete the ligand. At every step of the reconstruction a specified number of optimal partial solutions are selected for the next step. As soon as the ligand is reconstructed, the selected poses are scored.

FlexX uses an incremental construction algorithm to place ligands in the active site. The FlexX docking method can be divided into three parts, which start with the sampling of the conformational space of the ligand, modeling of the protein-ligand interactions, followed by scoring the protein-ligand interactions. The scoring function used by FlexX has its roots in a modified version of LUDI.

GLIDE 5.0.207 (Grid-based LIgand Docking with Energetics). Glide uses a series of hierarchical filters in order to place the ligand within the active-site region of the receptor.(Friesner, Banks et al. 2004) Glide approximates a complete systematic search of the conformational, orientational and positional space of the ligand by first doing an initial rough position and scoring run, followed by an optimization of the conformation on an OPLS-AA nonbonded potential grid. These initial steps narrow the search space with only a small number of candidate poses undergoing further refinement via Monte Carlo (MC) sampling. The best docked poses are selected using a combination of empirical and force-field-based terms. The receptor's shape and properties are represented on a precomputed grid by a set of fields that can provide progressively more accurate scoring of the ligand pose. Glide offers a full range of speed vs. accuracy

options, from a HTVS mode designed to handle million compound libraries to XP mode which can be used for the elimination of false positives and advanced scoring.

Glide uses a scoring function that is both an extended and modified version of Chemscore. Glide 2.5 docks explicit waters into the binding site in order to consider solvation effects. With the current version, water are not explicitly positioned in the final complex and the protein is only considered rigidly.

GOLD 4 (Genetic Optimisation for Ligand Docking). GOLD uses a genetic algorithm (GA) and supports full ligand flexibility and partial protein flexibility. Scoring of the poses can be done using GoldScore, ChemScore or a user defined score which allows the user to enhance the existing scoring functions. GOLD was made to be used in conjunction with a modeling program, and requires the input file to have the correct ionization and tautomeric states.

By default, GOLD rotates protein OH groups on serines, threonines and tyrosines, as well as terminal ammonium groups on lysines. However, it is worth keeping in mind that in some cases, more accurate results may be obtained by keeping the initial positions of the polar hydrogens, which can be done by running GOLD in "local optimization mode" with simplex optimization switched off. There are a number of parameters that are not accessible from GUI provided by GOLD's frontend that should be addressed in order to obtain the best results possible(Marcel, Jason et al. 2003).

GOLD also allows for water molecules to toggle on and off, which is a major advantage over most other current software. Unfortunately, some validation sets, including the sets that have been used to evaluate GOLD have protein complexes where most of the water molecules have been removed. Unexpectedly, the GOLD developers have found that this implementation does not improve the docking accuracy.

Surflex-Dock 2.415 (Tripos). The docking algorithm implemented in Surflex employs an idealized active site ligand called a protomol. This protomol is made of probes representing sites of potential hydrogen bonds and favorable hydrophobic interactions

with the protein which are merged into a negative picture of the binding site. This protomol is next used as a target to generate putative molecular poses or fragments. For this purpose, Surflex generates ligand fragments which reduces the conformational space that must be explored. The fragments are aligned onto the protomol probes. The remainders of the ligand's fragments are then docked started with each head fragment, the next fragment is aligned. The combined poses are refined and the poses scored. Surflex-Dock uses an empirical scoring function based on the Hammerhead docking system. The poses are scored using the Hammerhead scoring function which also takes part in the local optimization of the pose(Jain 2007).

FITTED 3.0. FITTED docks the ligands into flexible proteins using a matching algorithm enhanced genetic algorithm. Protein side chains, protein backbone conformations, ligand torsion angles as well as water molecule positions are all optimized through a Lamarckian/Darwinian evolutionary process (Corbeil, Englebienne et al. 2007). Each protein / water / ligand complex is described using a single chromosome on which evolution operators are applied. To select poses, a complex consensus docking based on various scores of increasing precision is used. Through this process, each pose is sequentially evaluated for its position in the binding site, its match with interaction sites and its force field-based score. The selected ligand poses are then further optimized using a Fletcher-Reeves conjugate gradient minimization. Not only can the water move but they can also be displaced during the docking/scoring process. Displacement is considered through the use of a specifically designed energy function which turns of the water when interpenetrating with any ligand atom.

Lead Finder 1.1.11 build 58.0. As does FITTED, Lead Finder combines the classical GA with local optimization procedures and exploitation of the knowledge generated during the docking process. The scoring functions are based on a molecular mechanics function that accounts for different types of energy contributions that are scaled with empiric coefficients. This scoring function can be tailored for specific tasks which include prediction of binding energies, energy-ranking docked ligand poses and for rank-ordering active and inactive compounds (Stroganov, Novikov et al. 2008).

PLANTS 1.1. The Protein Ligand ANT System (PLANTS) when given a protein structure, a ligand structure and a scoring function, finds a low energy conformation in the proteins binding site that corresponds to the global minimum of the scoring function. The algorithm for docking is based on a class of stochastic optimization algorithms which is suitably called, ant colony optimization (ACO) (Korb, StuÌˆtzle et al. 2009). ACO was developed by observing the real life behavior in ants which allow them to find the shortest path between their nest and their food source. In this artificial case, artificial pheromone trail information is modified in subsequent iterations in order to generate conformations with higher probability.

3.4 General Protocol for Comparative Virtual Screening

3.4.1 Preparation of the Receptor and Ligand Sets

Each of the proteins was prepared following previously reported procedures (Corbeil, Englebienne et al. 2007; Corbeil and Moitessier 2009; Englebienne and Moitessier 2009) and saved in the appropriate format for each of the docking programs.

3.4.2 Preparation of the Ligand Sets

For each receptor, the DUD provides the active ligands and the decoy ligands in separate .mol2 files. The .mol2 files were concatenated and loaded intro Maestro where the native ligands from the co-crystallized receptors were appended to the list or actives and decoys. In order to simplify the analysis of the results, the ligands and the actives were docked separately. The separate ligand and active files were converted to the required input file for any given VS tool as required. The addition of the co-crystallized ligands allows the VS to provide self-docking results in addition to enrichment results. The addition of the co-crystallized ligands into the VS made it necessary to check that duplicate active ligands were not being added into the set, thereby biasing the results. It is also of interest to see whether these native ligands would rank highly in the ordered list.

Due to CPU time constraints, it was decided to uniformly shrink the decoy/ligand set to have a total of no more than 1000 unique molecules. The ligand to decoy ratio was set to

40 ligands for every 960 decoys plus tautomers. A script was used which randomly chose "n" molecules from a larger set. An option to have the user specify the seed used for the random number generator was also implemented in the script, so that the random generation of molecule sets could be reproduced, while still being random. Due to the number of tautomers included in the DUD mol2 files, the script was written so that if a tautomer was encountered, all of the tautomeric states of that molecule would be written into the subset. This resulted in decoy/ligand sets that contained 1000 unique molecules, while containing over 1000 structures due to the tautomers. In cases such cases where less than 40 actives or 960 decoys were available from the DUD set, a smaller subset was produced.

3.4.3 Metrics for measuring accuracy

The conclusions of the study will depend on how success is evaluated. A commonly used metric is the Enrichment Factor (EF). While this metric is simple to calculate, and easy to read, it is dependent on both the cutoff levels used (1%, 2%, 10%, etc.) and also dependent upon the ratio of actives to decoys (Figure 3.9).

Percent recovery (i.e., 47.6% of the known actives ranked in the top 5% of the library, Figure 3.9) is a metric that is easy to calculate and also provides an easy way to visualize the comparative efficiency of different VS results. One problem with these first two metrics is that two programs can provide identical values while one ranks the identified actives in the first 1% while the other ranks them in the top 10% (early recognition).

$$EF = \frac{Hits_{sampled}/N_{sampled}}{Hits_{total}/N_{total}}$$

$$EF = \{N_{total}/N_{sampled}\}\{Hits_{sampled}/Hits_{total}\}$$

$$EF' = \{50\%/APR_{sampled}\}\{Hits_{sampled}/Hits_{total}\}$$

Figure 3.8 Definition of enrichment factors.

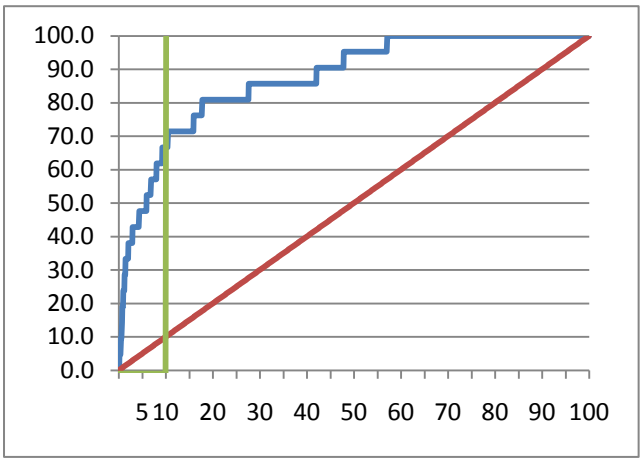

Figure 3.9 Measuring success. Active recovered (blue) vs. random recovery (red). Enrichment factor: EF=2.96 at 5%, EF=2.20 at 10%; AU-ROC=33.8 at 5%, AU-ROC=45.5 at 10%, AU-ROC=87.5 at 100%; Percent recovery: PR=47.6% at 5%, PR=66.7 at 10%.

Another important metric that is currently being used for the evaluation of the performance of ranking methods in VS is the area under the operating characteristic curve (ROC, Figure 3.9). ROC possesses many desirable statistical behaviors and it is claimed to be independent of the ratio of actives and decoys (Clark and Webster-Clark 2008). This claim has been called into question (Zhao, Hevener et al. 2009). However, ROC still remains a valuable metric and can provide a very useful method of measuring success, as long a good understanding of its limitations is taken into account (Zweig and Campbell 1993). The ROC can be represented as the fraction of true positives versus the fraction of false positives. The results of ROC can be adversely affected when actives are identified as decoys, and the opposite is also true. When considering a set of actives and decoys, and set of decoys that are too similar to the actives, the AU-ROC will be erroneously low. The major advantage of ROC over the other two metrics is its ability to capture early recognition.

In order to determine whether the ranking method (ROC in this work) is better than random ranking and to evaluate the impact of the selected datasets, a theoretical distribution can be derived by reasonable approximations, and bootstrapping is a useful method for deriving theoretical distributions. (Truchon and Bayly 2007). In the present work, in the case of a library with 40 actives and 960 decoys, 40 actives were randomly

picked (duplicates may be present and others may not be picked) and 960 decoys were randomly selected. The AU-ROC was computed and the process iterated 5000 times. Minimum and maximum AU-ROC were computed to cover 95% of the values.

The ligand to decoy ratio may have to be re-examined. A recent paper which discussed how VS studies are compared suggested that the ideal ratio should be used, depending on the size of the entire set and the metric(s) that will be used to evaluate the results.(Cavasotto and Abagyan 2004; Truchon and Bayly 2007) Having a large number of actives minimizes the intrinsic variance, but also leads to saturation. The area under the receiver operating characteristic (ROC) curve is often used to measure VS performance. While ROC possesses many useful statistical properties, it is dependent on the ratio of actives to decoys.(Clark and Webster-Clark 2008). The metrics used for measuring the success of a VS include the area under the accumulation curve (AUAC), the average position of the actives, analysis of variance (ANOVA), the Z-score, the enrichment factor (EF), the robust initial enhancement (RIE) and the Boltzmann-enhanced discrimination of receiver operating characteristic (BED-ROC). In this study ROC will be used as the measure of success.

3.5 Results and Discussion

3.5.1 A fair comparative study

The goal of many VS comparative studies that have been published have been relatively similar (Bissantz, Folkers et al. 2000; Kellenberger, Rodrigo et al. 2004; Chen, Lyne et al. 2006). In most simple terms, the goal of a comparative study of VS programs is to gauge the efficacy of a given program to rank order a large database of compounds or to enrich libraries in actives for a given protein target or set of protein targets. Some studies have been extremely thorough, such as that conducted by Warren *et al.* (Warren, Andrews et al. 2006*)*, which examined 10 docking programs and 37 scoring functions in collaboration with the developers.

Despite, and due to the number of comparative studies of this type that have already been conducted, many shortcomings have been identified, and no single study has been considered to be perfect. The challenges involved in designing a fair and effective comparative study which avoids biases and errors in interpretation of the results obtained from this type of study was examined by Kirchmair (Kirchmair, Distinto et al. 2008) and others (Jain 2008). VS tools can be exceedingly complex with numerous parameters that must be correctly matched for a given set of compounds being docked to a specific protein. Without expert knowledge in both the target being studied and the program being used to study it, it can be easy to obtain subpar results. This is one source of bias that must be avoided to achieve a fair comparative study.

3.5.2 Avoiding Bias

The most apparent potential pitfall of comparative studies which examine a number of docking tools to study a number of different targets lays in the fact that it is extremely difficult to use the software to its full potential for every given situation (Warren, Andrews et al. 2006). It is unfair to compare the accuracy of the docked poses of two programs if one program requires five minutes to predict the pose while the other program can dock several ligands in the same time frame. The former would fit the analogy of having a "level playing field". The more difficult problem is illustrated by another analogy. This analogy would be having a soccer coach in charge of the hockey team, the soccer team, the swimming team and the football team. In order to avoid biasing the results towards the soccer team, several methods can be implemented to avoid biasing the results of the comparative study.

To reduce the bias towards our own docking program FITTED and objectively make comparisons and draw fair conclusions, we decided to use only the default parameters with FITTED. Also, when possible, consultation with the developers of the software was used to determine whether the software is being used to its full potential. It is worth mentioning that a number of parameter sets were used with the other seven programs while only one set of parameters was used with FITTED.

Another important factor that must be considered is how the results are compared across programs. A number of metrics have been used to compare the results of VS studies. In a

paper by Truchon (Truchon and Bayly 2007) several metrics commonly used to measure success in VS are examined and a new metric based on the ROC metric is proposed. The new metric, BEDROC, was developed to address the "early recognition" problem. Clark (Clark and Webster-Clark 2008) also suggested modifications to the ROC metric. The first modification to account for the "early recognition" problem, and a second modification which entails weighting each active based on the size of the lead series to which it belongs.

Choosing a suitable database for the evaluation of VS tools is also of great importance. A number of public databases of actives and decoy compounds have been used by previous comparative studies. When choosing a set of compounds used for the testing of VS tools several factors must be considered. The first one is that the database must be suited to what is being studied. One database may be more suitable if one is determining the ability of a program to find hits, while another may be more suitable for the hit-to-lead process (Huang, Shoichet et al. 2006).

3.6 Evaluating the impact of protein flexibility, water molecules and selected docking program

Several different run modes were used in the virtual screening of the proteins being studied for each of the programs. The proteins were considered with all crystallographic waters removed (where applicable), herein described as "dry" proteins. When the proteins had conserved crystallographic waters that were deemed to be important in binding and were kept, they were referred to as "wet" proteins. In order to simulate displacement of the water molecules, the best score for each ligand computed in these first two modes was selected and the ligand re-ranked. In this case, all the waters are kept or all are removed. This mode was called displaceable. Certain programs (FITTED, GOLD, PLANTS) have the ability to displace some water molecules while keeping other while docking. This approach represented a second type of displaceability.

A database containing all 21760 entries was built and used to analyze the data. In order to evaluate the error associated with each of the run, a bootstrapping approach was used. With this approach, the statistical significance can be assessed. This error is however not given in all the figures for clarity purpose and will be discussed when important.

Program	Protein	PDB ID	Run Mode	CutOff	Result Type	Result
FiTTED	CDK2	1dm2	Dry	2	avg	19.5
eHiTS	CDK2	1dm2	Dry	2	avg	11
FlexX	CDK2	1dm2	Dry	2	avg	10.4
Glide	CDK2	1dm2	Dry	2	avg	7.5
LeadFinder	CDK2	1dm2	Dry	2	avg	6.8
GoldGS	CDK2	1dm2	Dry	2	avg	5.5
GoldCS	CDK2	1dm2	Dry	2	avg	5.5
PLANTS	CDK2	1dm2	Dry	2	avg	4.8
Surflex	CDK2	1dm2	Dry	2	avg	4.2

Table 3-2 Subset of the database

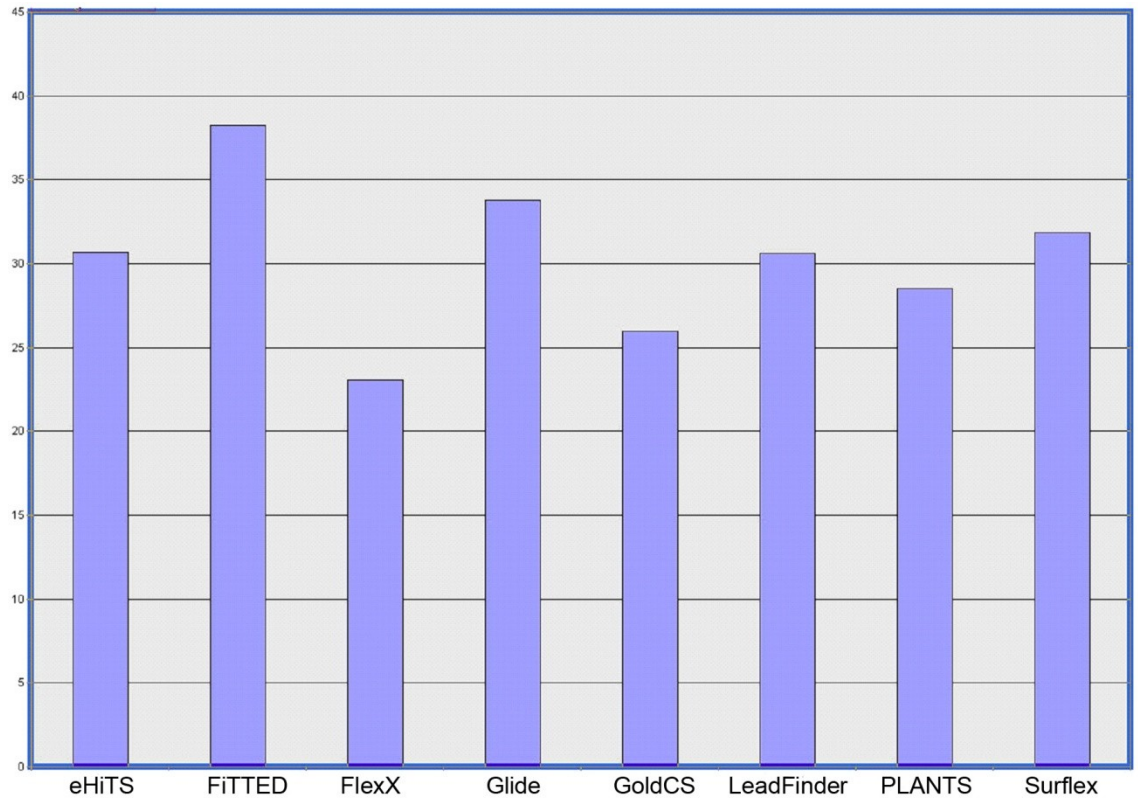

Figure 3.10 Overall results – All programs – All proteins averaged

An overall trend in the efficacy of VS is shown above (Figure 3.10) which factors in all run modes across all proteins for each program. This table shows the overall ability at of each program at a cut off of 5 to enrich a given set of actives and decoys for each of the proteins described earlier. As shown by this graph, FITTED and Glide hold a significant advantage overall in rank ordering of actives and decoys over the proteins studied when averaged across all run-modes. However, we wish to point out once more that this is valid only for the set of proteins we have considered and that much larger sets are needed to truly rank programs. The effects of protein dependence, run-modes and cut-offs will be further discussed and highlighted in the examples that follow.

3.6.1 Impact of the presence of water molecules

The inclusion of water molecules in proteins undergoing virtual screening is an important topic that has not been fully addressed to date. For example, a VS study involving the docking programs, FRED, QXP/FLO and Glide was conducted on cyctochrome c peroxidase (CCP) included the evaluation of the effect of the inclusion of HOH308 in docking runs of the aforementioned programs (Deng and Verlinde 2008). In the case of QXP/FLO and FRED, the inclusion of HOH308 resulted in a small but significant drop in AUC-ROC values, while in Glide, no significant change was observed with the inclusion/exclusion of HOH308. In the current study, a more thorough study was of the effect of water in VS was completed, as six of the seven proteins possess conserved water and seven programs were used. In Figure 3.11, the effects of keeping, discarding and allowing water molecules to be displaceable when docking to rigid protein are illustrated.

Depending on the protein studied, the presence or absence of water molecules can or not impact the programs' accuracy. As shown in Figure 3.11, in the case of ER, the conditions used for the VS did not significantly affect the results of any of the programs. This seems to be an anomaly, because as we will see in later examples, the conditions and individual proteins of a family can drastically affect the ability of a program to give good results. In stark contrast to the example of ER, the run mode had a significant impact in

the case of HIVP. While both ER and HIVP have crystallographic waters that were kept considered during the VS runs, the results obtained in the HIVP runs clearly depend on how they are treated. This dependence on water treatment is very much program dependent in this case. When the results of FITTED and PLANTS are examined, the dry case is the worst run mode, while the dry cases are the best for Glide and Surflex.

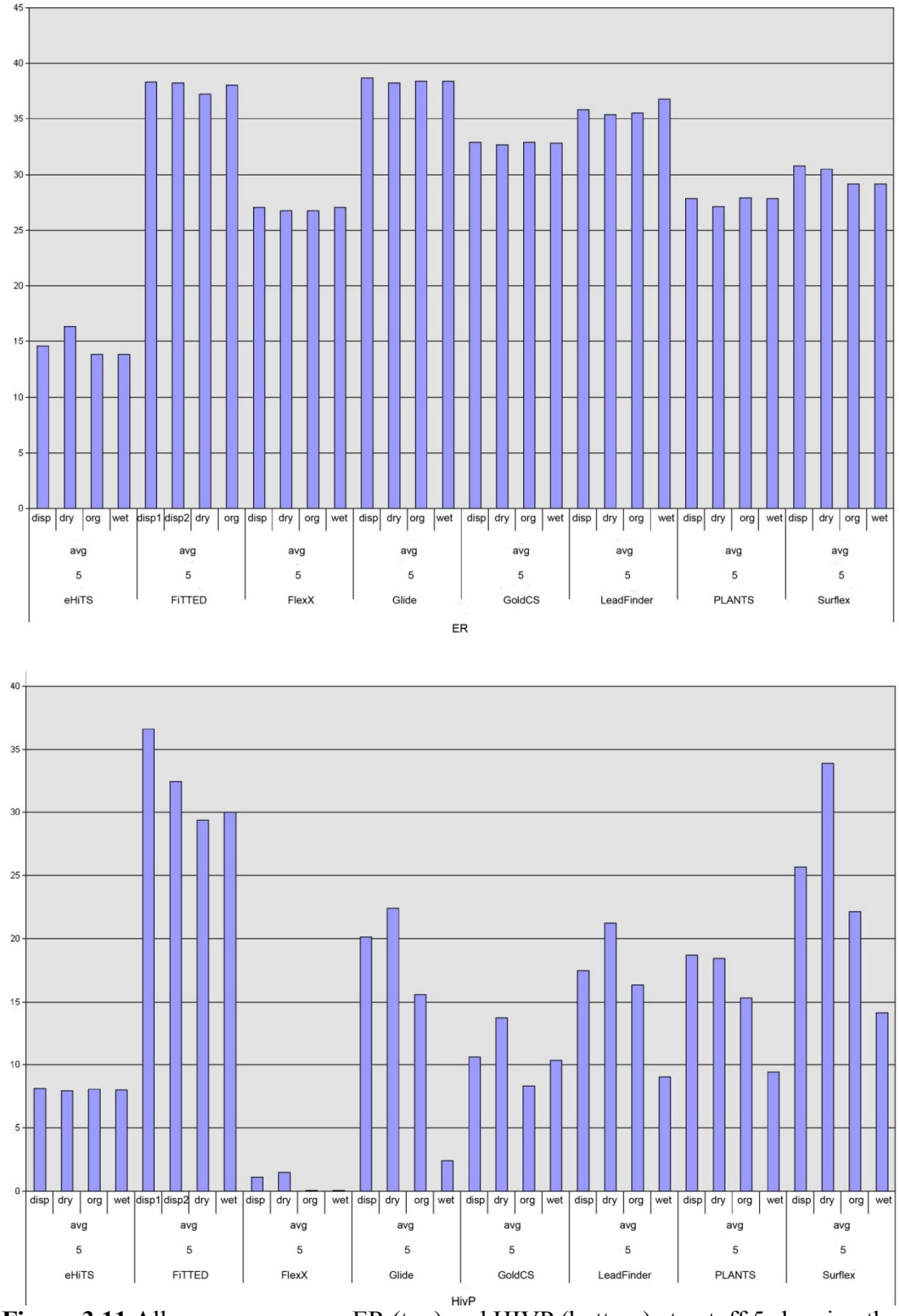

Figure 3.11 All programs run on ER (top) and HIVP (bottom) at cutoff 5 showing the effects of run-mode.

3.6.2 Program and water dependence

Glide and FITTED are two programs that perform the best overall across all the proteins used in this study. Interestingly, these two programs have very different behavior depending on whether a wet protein is used or not. Figure 3.12 shows that in all cases the use of a wet protein is beneficial when FITTED is used, while the effect of including waters in Glide is nearly always detrimental.

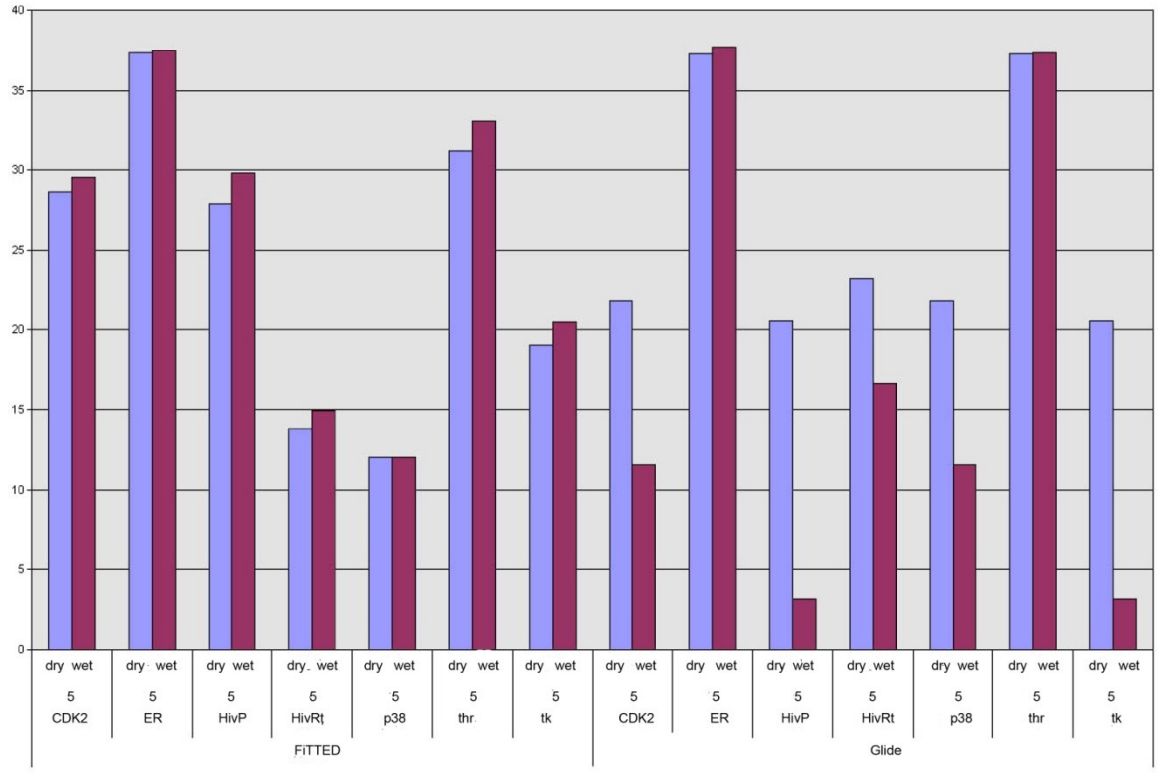

Figure 3.12 Water dependence across all proteins using FITTED and Glide

3.6.3 Protein Flexibility

When comparing flexible runs to those run rigidly, flexibility always affords an advantage over the rigid runs, sometimes a small advantage, but always an advantage overall (Figure 3.13). While this chart shows an overall effect of flexible versus rigid runs

over all proteins studied, it is far more enlightening to look at individual cases where the effects are more pronounced. One such case is shown in Figure 3.14 which shows that overall, the flexible mode gave better results than the rigid modes and that the effects of the runmode was program dependent. For example, Surflex, when run rigidly or flexibly gave much better results with the "dry protein" compared to either displaceable waters or the "wet protein" however the for the other programs, little difference was observed.

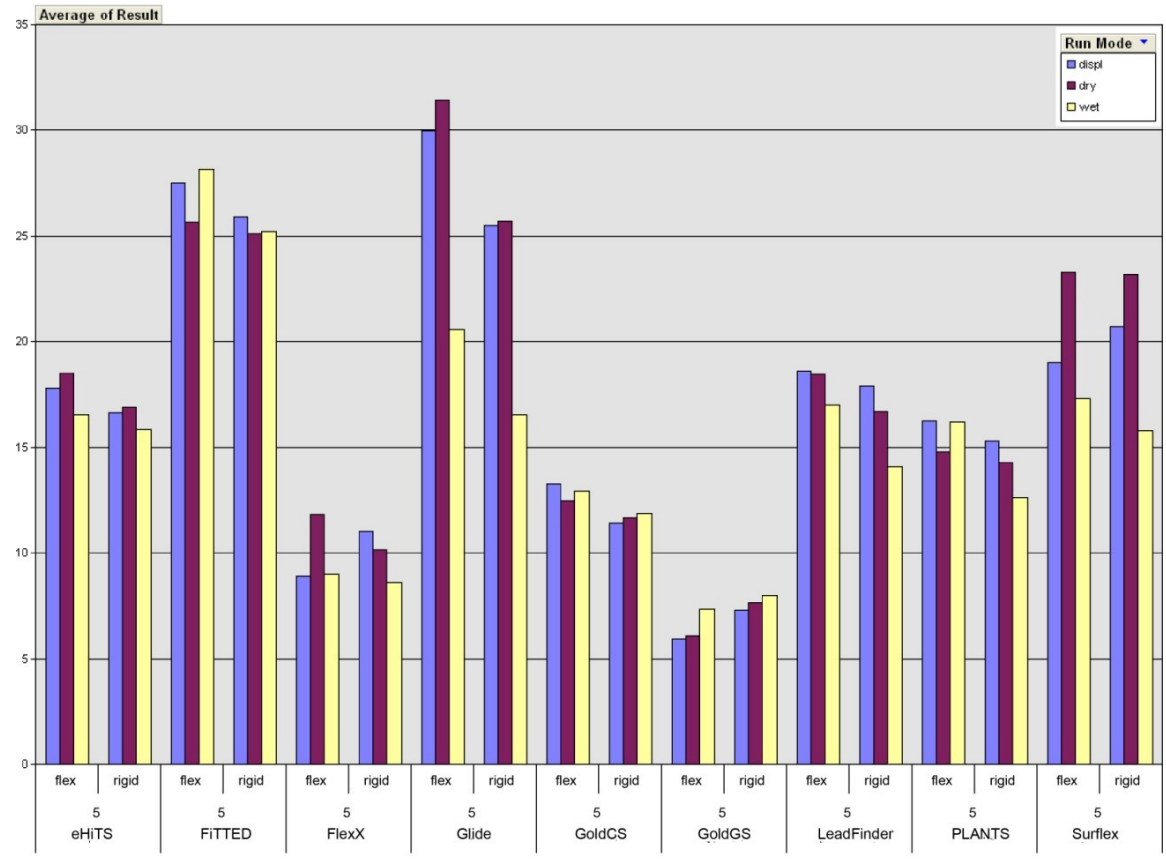

Figure 3.13 All programs – Flexibility versus rigid overall

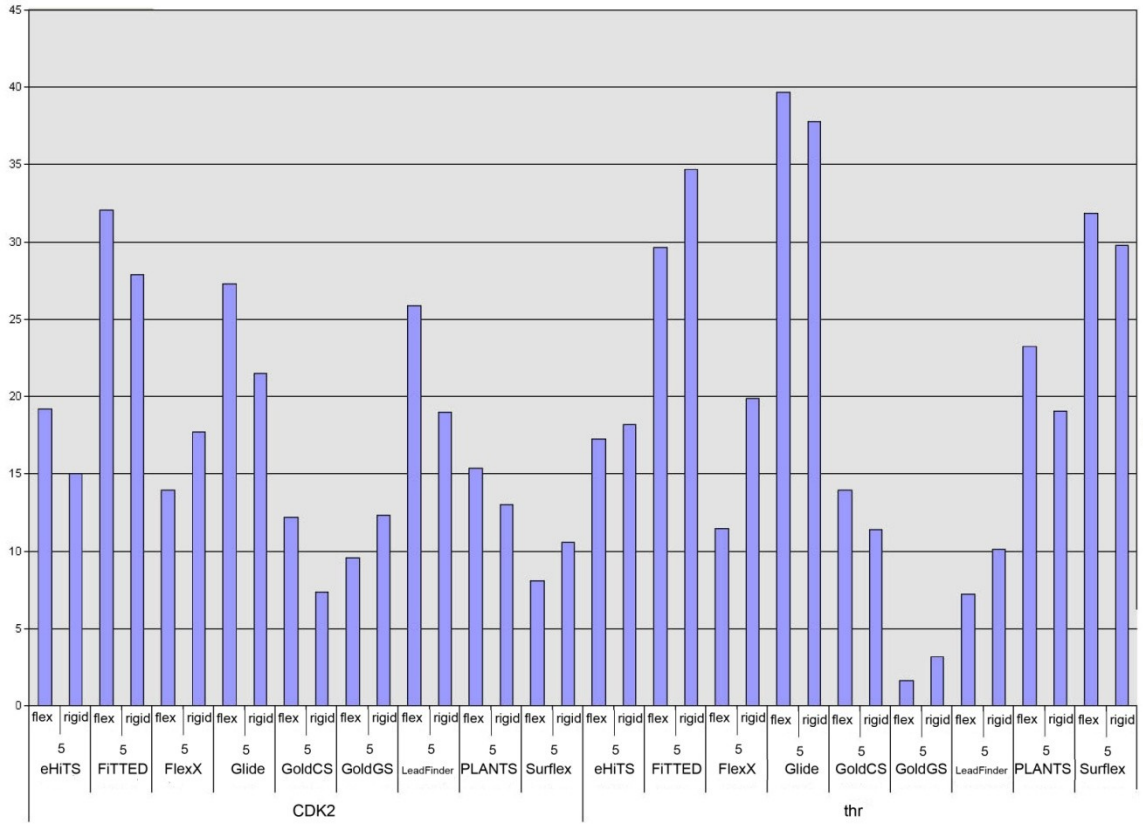

Figure 3.14. All programs run on CDK2 and Thrombin showing protein dependence on whether the protein was run flexibly or rigidly.

When CDK2 and THR are run by all programs, protein dependence, which is related to flexibility/rigid is seen (Figure 3.14). In CDK2, the flexible runs are always better while in thrombin the rigid are always better. These results highlight the need for choosing the correct method for running a virtual screen (rigid/flexible), which can be just as important as choosing the right program for the job.

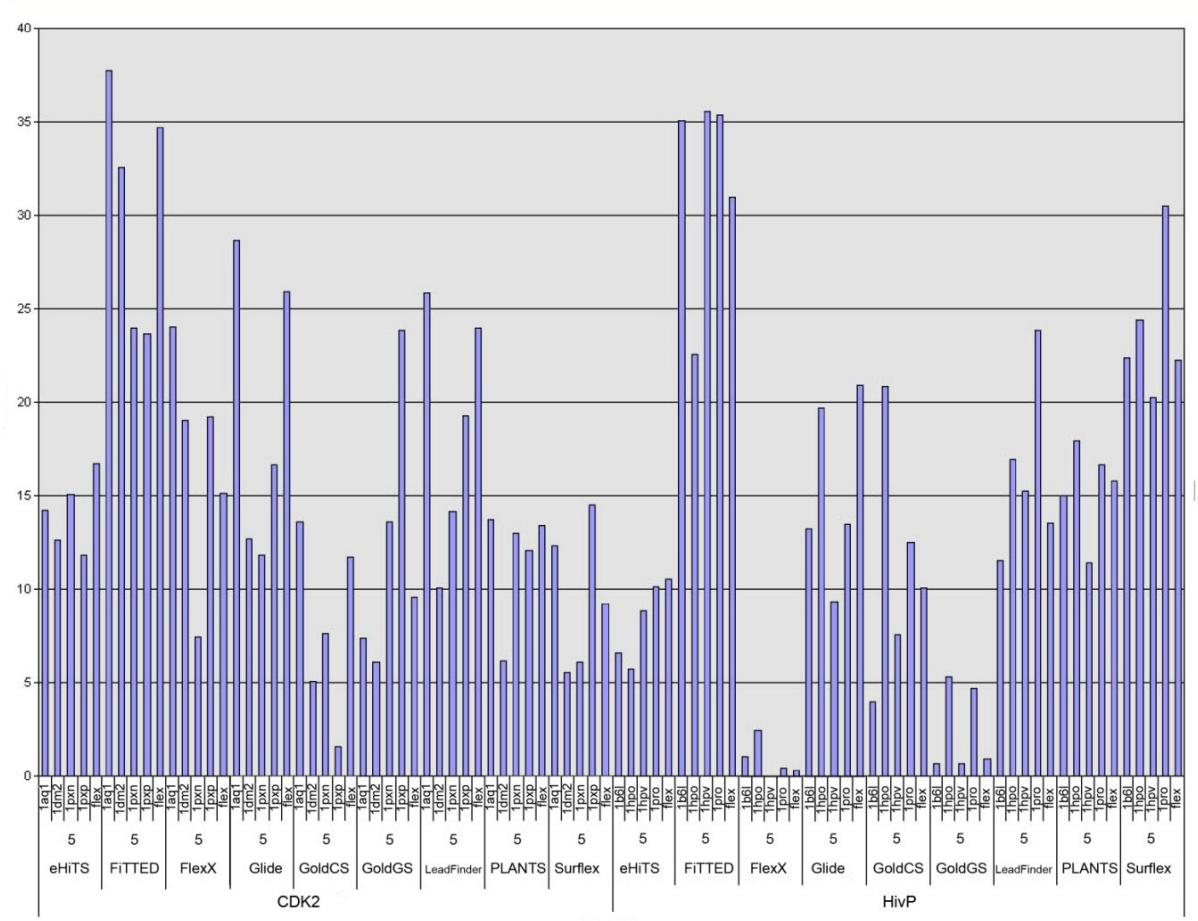

Figure 3.15 Flexibility vs. Rigid averaged across all proteins

The results obtained from this study shows that for all of the programs in question the flexible runs generally generated some improvement in the results. However consider the case illustrated in Figure 3.15. In this case, CDK2 run with flexibility is advantageous and in the case of HIVP, flexibility is disadvantageous, showcasing the protein dependency on flexibility.

3.6.4 Protein Dependence

Figure 3.16 shows Plants and Surflex run on ER, p38 and TK in which protein dependence displayed. The effects of running the proteins wet versus dry across all proteins are especially apparent in TK and HIVP.

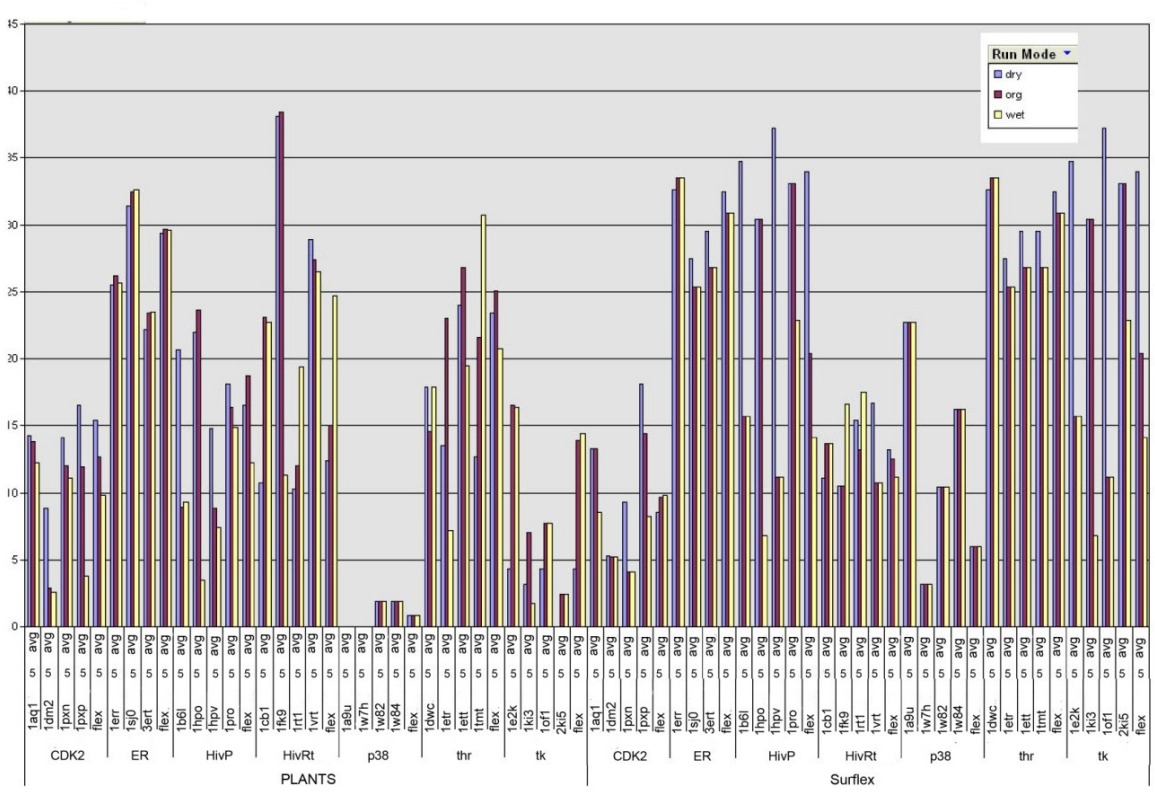

Figure 3.16. Protein Dependence - water - Shown by Plants and Surflex on ER, p38 and TK

3.6.5 Program dependence

Figure 3.17 illustrates how the accuracy of programs can vary from one protein to another. All programs do well for ER except eHiTS while all programs do badly for p38 except eHiTS. TK is done very well by some programs, and very badly by others, giving an telling example of protein dependence, thus Figure 3.17 provides a prime example of how certain programs can excel well beyond its competitors in one case for a given protein target, while yielding far worse results in other protein targets.

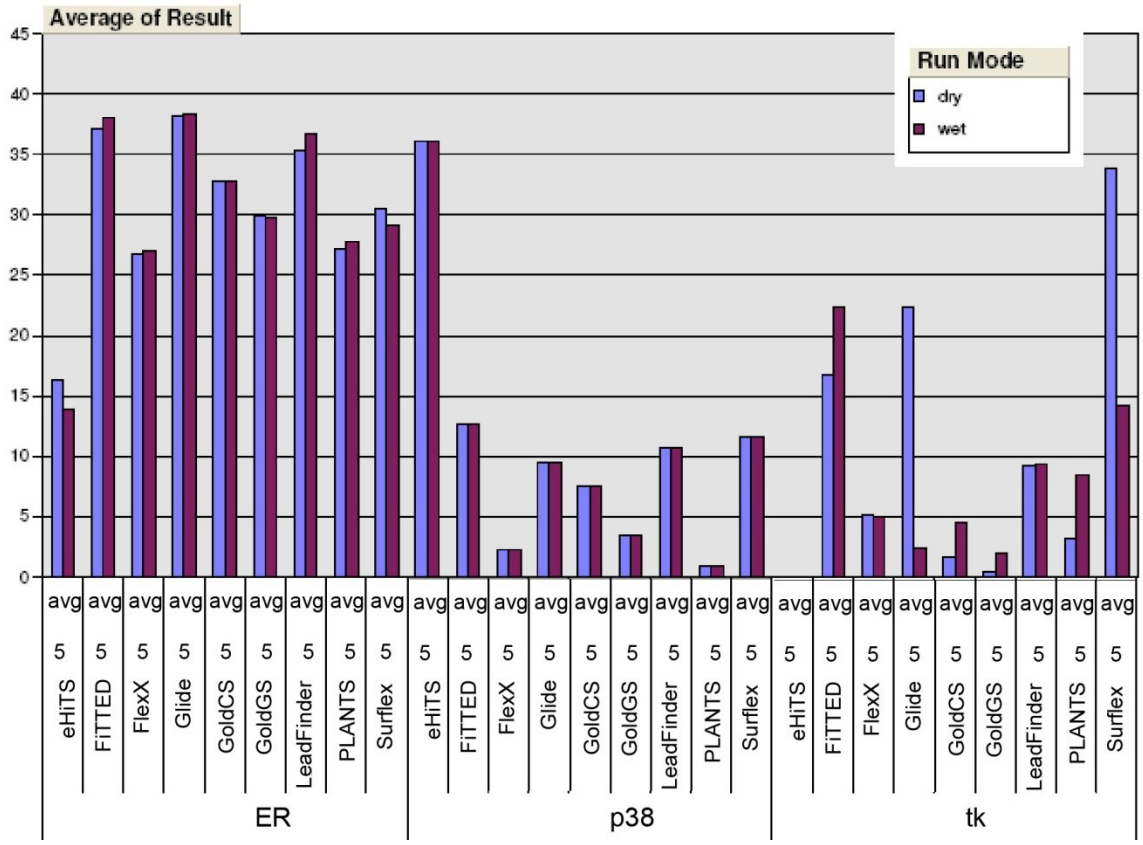

Figure 3.17. Protein Dependence – Protein – All programs on ER/p38/TK

The results in Figure 3.17 show that for ER, P38 and TK, the same trends seen for the rigid runs are seen in the flexible runs, therefore showing an example where flexible runs versus rigid runs provide essentially equivalent results across programs for the proteins in question.

3.7 Conclusions

After examining the overall results from the VS study and specific examples, several recurring themes become clear. The accuracy of a VS will most likely always be protein, run-mode and program dependent. In some cases (such as ER) the run mode dependence will be minimal, and the program dependence will be pronounced. In this type of case, eHiTS is clearly one program that should not be used. However, if the protein undergoing study is P38, eHiTS would be the clear choice of programs.

Running the proteins with flexibility is overall advantageous for all proteins studied. However, this is not categorically true, as was shown in the example of CDK2 and thrombin. While the CDK2 flexible runs were always better while in thrombin the rigid runs were always better.

The importance of the inclusion and use of waters is also an interesting point of discussion and is very much program dependent. We have found that for each protein, Glide generally produces better results with dry proteins, while FITTED produces better results with wet proteins.

The overall conclusion from the data obtained from this study suggests protein dependence and program dependence can range from insignificant to highly significant, and therefore a high level of knowledge of both the program and protein much be used, when available prior to carrying out a VS. Unfortunately when studying a new protein target, by definition, very little may be known about the protein and how key crystallographic waters or flexibility may affect binding. In these cases, the use of knowledge based on proteins with high amounts of homology with the protein in question should be used, as well as a program that has shown good efficiency with similar proteins.

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