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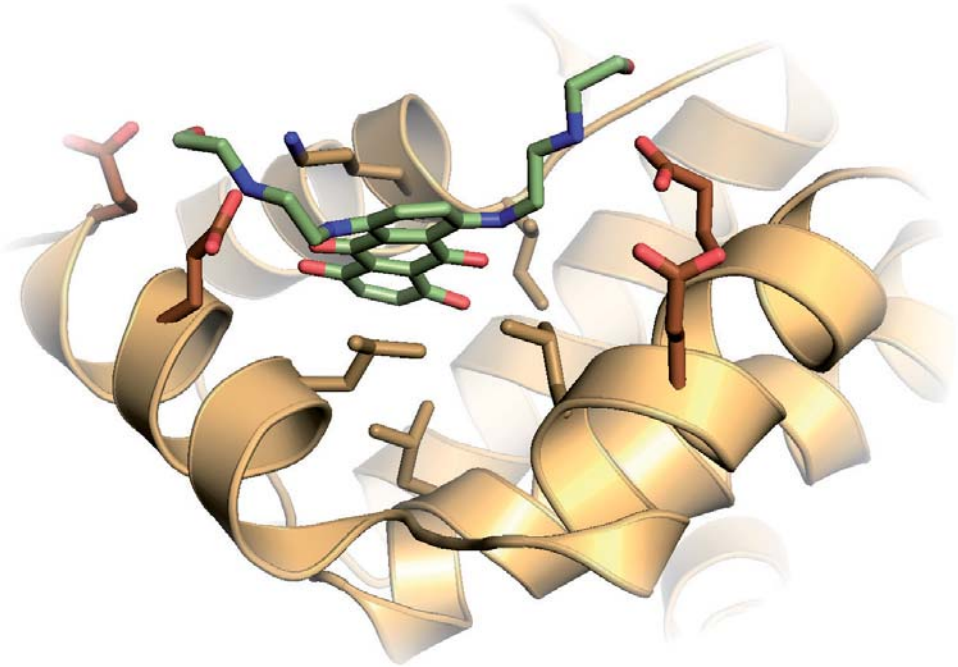
221

Elisa Nurminen

Rational Drug Discovery

Structural Studies of Protein-Ligand Complexes



JYVÄSKYLÄN YLIOPISTO

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UNIVERSITY OF JYVÄSKYLÄ

JYVÄSKYLÄ 2011

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"Science is simply common sense at its best."

- Thomas Huxley

ABSTRACT

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Rational drug discovery is one possible strategy for the discovery of novel compounds for pharmaceutical industry. The availability of experimentally determined 3D protein structures is important in rational drug discovery. Rational drug discovery is a combination of computational and experimental methods. In this thesis a variety of methods involved in drug discovery have been employed to study three separate proteins important for human health 1) Vascular adhesion protein 1 (VAP-1), 2) filamin A and 3) T-cell protein tyrosine phosphatase (TCPTP). VAP-1 is an enzyme which converts biogenic and xenobiotic amine compounds into corresponding aldehydes. The reaction products are toxins in high concentrations and are responsible for, e.g. diabetic complications. The design of subtype-selective VAP-1 inhibitors has been problematic since the members of the amine oxidase family have overlapping substrate specificities. In this thesis a set of hydrazine molecules was used as a tool to understand the subtype-selectivity of VAP-1. New insight into the flexibility of the VAP-1 ligand-binding pocket was also acquired. Cystic fibrosis transmembrane conductance regulator (CFTR) is a chloride ion channel. Mutations in CFTR cause cystic fibrosis. Interaction of CFTR with filamins is required for the proper functioning of CFTR. In this thesis the subdomain selectivity of filamin A domains was studied using sequence alignment and structure based sequence alignment. T-cell protein tyrosine phosphatase (TCPTP) is a potent anti-cancer target. Novel activators of TCPTP have been identified but the binding site for them was unknown. In this thesis the binding site for activators was identified. The common factor in all three cases studied in this thesis is, that they all concern pathologies that currently lack proper treatments; diabetes, cancer, and cystic fibrosis.

Keywords: filamin; hydrazine; molecular dynamics; rational drug discovery; T-cell protein tyrosine phosphatase; vascular adhesion protein 1.

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Acknowledgements

YHTEENVETO (RÉSUMÉ IN FINNISH)

REFERENCES

LIST OF ORIGINAL PUBLICATIONS

The thesis is based on the following original publications, which will be referred to in text by their Roman numerals.

I Nurminen, E.M.., Pihlavisto, M., Lázár, L., Szakonyi, Z., Pentikäinen, U., Fülöp, F. & Pentikäinen, O.T. 2010. Synthesis, *in vitro* activity, and three-dimensional quantitative structure−activity relationship of novel hydrazine inhibitors of human vascular adhesion protein-1. J. Med. Chem. 53 (17): 6301-6315.

II Nurminen, E.M., Pihlavisto, M., Lázár, L., Pentikäinen, U., Fülöp, F. & Pentikäinen, O.T. 2011. Novel hydrazine molecules as tools to understand the flexibility of vascular adhesion protein 1 ligand binding site: Toward more selective inhibitors. J. Med. Chem.

III Playford, M.P.*, Nurminen, E.*, Pentikäinen, O.T., Milgram, S.L., Hartwig, J.H., Stossel, T.P. & Nakamura, F. 2010. Cystic fibrosis transmembrane conductance regulator interacts with multiple immunoglobulin domains of filamin A. J. Biol. Chem. 285 (22): 17156-65.

IV Nurminen, E.M., Mattila, E., Ylilauri, M., Määttä, J., Pentikäinen, U., Ivaska, J. & Pentikäinen, O.T. 2010. Mitoxantrone activates T-cell protein tyrosine phosphatase (TCPTP) *via* binding to a hydrophobic groove. Submitted manuscript.

* Equal contribution to the work.

RESPONSIBILITIES OF ELISA NURMINEN IN THE ARTICLES OF THIS THESIS

Articles I-II:

Elisa Nurminen is responsible for the 3D QSAR model building, molecular docking, and molecular dynamics simulations. Elisa Nurminen has participated in the writing of articles and has prepared most of the figures.

Article III:

Elisa Nurminen is responsible for the sequence alignment and structure based sequence alignment of the immunoglobulin like domains of filamin A.

Article IV:

Elisa Nurminen has cloned, expressed and purified proteins, done the ITC and DSF experiments, and modeling. Elisa Nurminen has written the article with other authors and prepared most of the figures.

All the studies were performed under supervision of Olli Pentikäinen and Ulla Pentikäinen.

ABBREVIATIONS

2HP	2-hydrazinopyridine
3D QSAR	Three dimensional quantitative structure-activity relationship
AO	Amine oxidase
CAO	Copper-containing amine oxidase
CFTR	Cystic fibrosis transmembrane conductance regulator
CoMSIA	Comparative molecular similarity indices analysis
DAO	Diamine oxidase
DSF	Differential scanning fluorimetry
EC	Enzyme commission
ECAO	*E.coli* amine oxidase
FAD	Flavin adenine dinucleotide
FLN	Filamin
GLUT4	Glucose transporter type 4
Ig-like	Immunoglobulin-like
ITC	Isothermal titration calorimetry
LO	Lysyl oxidase
MAO	Monoamine oxidase
MAOI	Monoamine oxidase inhibitor
MD	Molecular dynamics
PDB	Protein Data Bank
RAO	Retina-specific amine oxidase
RTK	Receptor tyrosine kinase
SSAO	Semicarbazide-sensitive amine oxidase
STAT	Signal Transducer and Activator of Transcription
sVAP-1	soluble VAP-1
TC37	Constitutive active 37 kDa form of TCPTP
TC45	45 kDa isoform of TCPTP
TC48	48 kDa isoform of TCPTP
TCPTP	T-cell protein tyrosine phosphatase
TPQ	Topaquinone
VAP-1	Vascular adhesion protein 1
WT	Wild-type

1 INTRODUCTION

Proteins are important biological macromolecules and their versatility is striking. For example, actin is responsible for constructing the intracellular network of supporting filaments, and protrusions called filopodia that facilitate the movement of cells. Some hormones are proteins, like insulin, which is composed of 41 amino acids and secreted by pancreatic β cells in the islets of Langerhans. Many cells express chaperones, which are proteins that help other proteins fold correctly during their synthesis and aid them retaining their native conformation when stressed. Cellular signaling is a process that occurs *via* numerous receptor proteins.

Enzymes are proteins that serve as biocatalysts. They take part in chemical reactions and lower the reaction's activation energy. In the end the enzyme is restored and it can continue to function as a catalyst. Enzymes can be classified into six classes: 1) oxidoreductases, 2) transferases, 3) hydrolases, 4) lyases, 5) isomerases, and 6) ligases. The cofactor is a special part of an enzyme that takes part in the enzymatic reaction. It can be an inorganic group like metal ion, an organic group, or a native amino acid that has been post-translationally modified (e.g. topaquinone in vascular adhesion protein 1, VAP-1).

The basis of protein function is their ability to interact with other proteins or with small molecular compounds. Proteins are also the factors behind many diseases. Diseases can be treated by designing small molecular ligands targeted to the desired proteins. Drug discovery is the first step in the development of pharmaceuticals and it is based on a combination of computational and experimental methods. Rational drug discovery (Fig. 1) often begins with the identification of the target protein. The acquisition of the target protein's 3D structure is the next priority. If there is no structure available, it is also possible to study the protein by using sequence alignment and homology modeling.

When the 3D structure of target protein is available, the visualization of the structure may reveal possible binding sites. Small molecules can be docked inside the binding sites and the interactions between the protein and the ligand can be reviewed. In this way, valuable information about the possible binding mode is obtained and better ligands can be designed accordingly. Molecular

dynamics (MD) is used to understand the interactions between protein and co-crystallized/docked ligand, and protein flexibility. By combining computational methods in Fig. 1, it is possible to design mutations, which are used, for example, to confirm the importance of certain amino acids to the binding of ligands. Drug discovery is an iterative process, and repetition of modeling, synthesis, and *in vitro* testing is required for the achievement of a successful outcome.

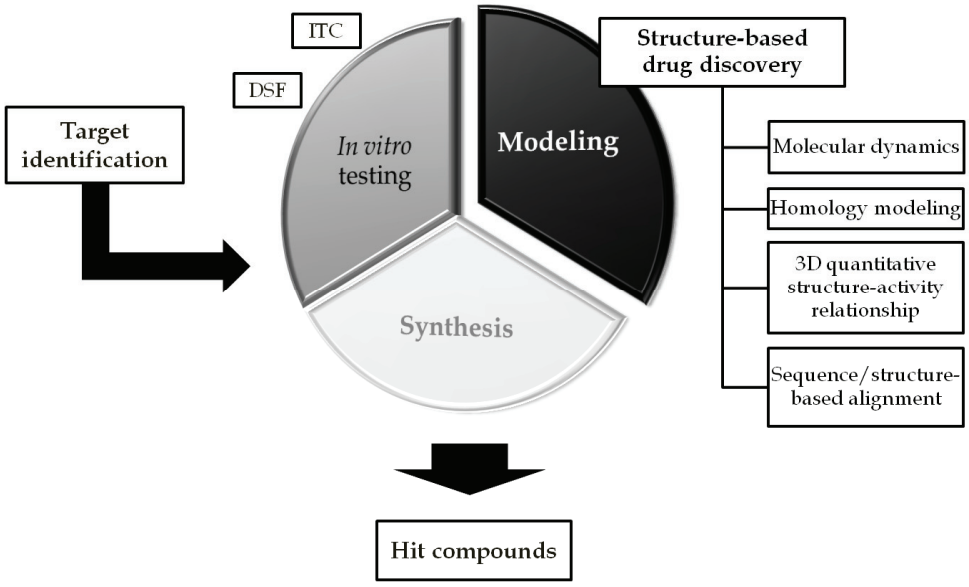


FIGURE 1 The pipeline of structure-based drug discovery.

2 REVIEW OF THE LITERATURE

2.1 Amine oxidases

Amine oxidases (AOs) are enzymes that degrade amine compounds into corresponding aldehydes with concomitant production of hydrogen peroxide and ammonia (Fig. 2). Amine oxidases emerged early in evolution and, in addition to vertebrates and plants, they can be found in species such as the archaebacterium *Methanosarcina barkery*, the bacterium *Escherichia coli,* and in the fungus *Aspergillus niger*.

AOs can be divided into two structurally distinct classes (Fig. 3): 1) Flavin adenine dinucleotide (FAD, Fig. 4.) containing amine oxidases and 2) copper-containing amine oxidases (CAOs). Monoamine oxidases (MAOs, enzyme commission number (EC) 1.4.3.4) belong to the former group and are located on the outer membrane of the mitochondrion. Two isozymes of MAO are present in humans: MAO A and MAO B. Their role is to degrade amine neurotransmitters such as serotonin and dopamine. MAOs are linked to diseases like depression (Meyer et al. 2006) and Brunner's syndrome (Brunner et al. 1993). Polyamine oxidases (EC 1.5.3.13-17) are also members of FAD containing AOs.

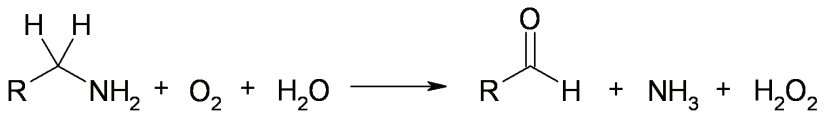


FIGURE 2 The general reaction catalyzed by amine oxidases. Substrate molecule, molecular oxygen, and water are converted to aldehydes, ammonia, and hydrogen peroxide.

Distinctive to CAOs is that they can be inhibited by the carbonyl reagent semicarbazide. The term semicarbazide-sensitive amine oxidase is, however, limited to denote only those amine oxidases that are sensitive to semicarbazide

and show little activity toward diamines, but relatively high activity toward benzylamine.

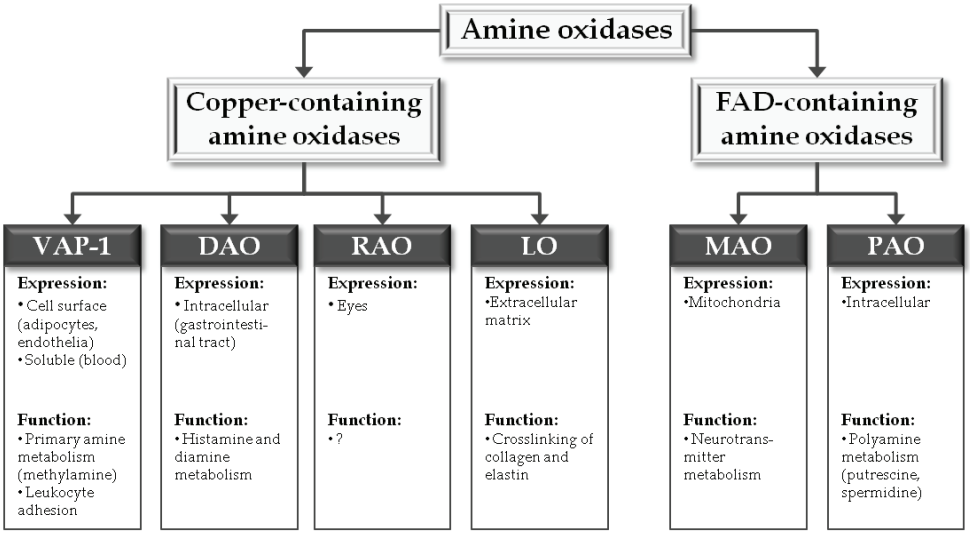


FIGURE 3 Classification of AOs. Amine oxidases are classified as CAOs and FAD-containing AOs. For each AO the expression site and function has been introduced.

In humans, four genes encode CAOs: 1) *AOC1* (diamine oxidase, DAO, EC 1.4.3.22), 2) *AOC2* (retina-specific amine oxidase, RAO), 3) *AOC3* (VAP-1/SSAO, EC 1.4.3.21), and 4) *LOX* (Lysyl oxidase, LO, EC1.4.3.13). The cofactor in the first three CAOs is a post-translationally modified tyrosine residue called topaquinone (TPQ) (Fig. 4) (Janes et al. 1990) whereas in LO the cofactor is a crosslink of tyrosine and lysine side chains, a lysine tyrosylquinone (Wang et al. 1996a). All CAOs have a copper ion coordinated to three conserved histidines, which is needed for the formation of cofactors (Janes et al. 1990, Wang et al. 1996a) and also for the reoxidation of the TPQ (Mure et al. 2002).

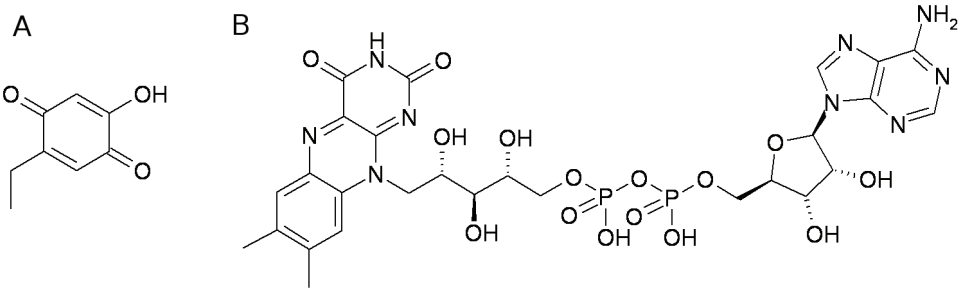


FIGURE 4 Amine oxidase cofactors. A. The structure of TPQ. B. The structure of FAD.

2.1.1 Vascular adhesion protein 1

VAP-1 was cloned in 1998 by Smith et. al. (Smith et al. 1998) and it was first identified as a protein participating in leukocyte adhesion to endothelium (Salmi & Jalkanen 1992). Later it was realized that it actually is the enzyme SSAO, which had been identified in the late 1950s (Bergeret et al. 1957).

VAP-1 is a 180 kDa protein (Salmi & Jalkanen 1996) and it has six potential N-glycosylation sites, all asparagine residues. The glycosylation is varied depending on the expression site of VAP-1 (Jaakkola et al. 1999). The glycosylation sites at the apical side of VAP-1 are important for the enzyme's adhesive task (Maula et al. 2005). VAP-1 has a ^{726}RGD^{728} motif that in collagen and fibronectin mediates their binding to integrins. In the crystal structure of VAP-1 the RGD motif is clearly visible and has adopted a prominent finger-like conformation (Airenne et al. 2005).

The biologically active unit of VAP-1 is a homodimer; each subunit is 90 kDa apiece. VAP-1 is largely expressed in adipocytes, in vascular endothelial cells, and in smooth muscle cells (Jalkanen & Salmi 2001) but not in the brain (Boomsma et al. 2005). VAP-1 has a membrane bound domain with an extracellular domain outside the cell membrane. The soluble form of VAP-1 (sVAP-1) is found in serum and it originates *via* proteolytic cleavage of membrane bound VAP-1 from endothelial cells or adipocytes (Stolen et al. 2004, Abella et al. 2004). The sequence identity of VAP-1 and sVAP-1 is 80 % and their kinetic activites are similar. Thus, it has been thought that their structures are also similar, even though sVAP-1 lacks the membrane anchor domain and the cytoplasmic tail of VAP-1 (Meszaros et al. 2000).

2.1.2 Roles of vascular adhesion protein 1

VAP-1 participates in lymphocyte recirculation in the inflammatory reaction (Marttila-Ichihara et al. 2006, Lalor et al. 2002, Kurkijärvi et al. 2001, Salmi et al. 2001, Salmi et al. 1993). VAP-1 interacts with leukocytes and granylocytes and facilitates their extravasation from blood vessels to the inflammation site in an L-selectin independent way (Salmi & Jalkanen 1992, Salmi et al. 2001, Tohka et al. 2001). In mammals, for example the domestic pig (*Sus scrofa domestica*), and the dog (*Canis lupus familiaris*), VAP-1 surface expression only occurs in inflammation sites, when VAP-1 is translocated to the luminal side of the endothelial cells from the intracellular compartments (Jaakkola et al. 2000). VAP-1 also controls the trafficking of glucose transporter type 4 (GLUT4) in adipocytes. Hydrogen peroxide, which is the product of AO-catalyzed reactions (Fig. 2), stimulates GLUT4 trafficking in a similar way to insulin (Enrique-Tarancón et al. 1998, Morin et al. 2001).

VAP-1 can degrade multiple amine compounds such as tyramine, benzylamine, methylamine, and aminoacetone (Deng & Yu 1999), and it is able to recognize amino acid side chains such as lysine (Wang et al. 1996b, Olivieri et al. 2007). It may possibly react with Arg293 in the recently identified binding partner Siglec-10 (Kivi et al. 2009). The adhesive function and the ability of

VAP-1 to recognize amines could be interlinked (O'Sullivan et al. 2003). The reaction products are predominantly toxic, such as formaldehyde and methylglyoxal derived from methylamine and aminoacetone, respectively (Yu & Zuo 1993, Lyles & Chalmers 1992), and increased enzymatic activity of VAP-1 can cause diseases. The reaction products do, however, have some functions, for example, in signaling, and therefore decreased levels of enzymatic activity can cause disease symptoms.

LO is responsible for cross-linking collagen and elastin, which is important for proper formation and stabilization of collagen fibrils and for the elasticity of the mature elastin (for review see, Smith-Mungo & Kagan 1998). VAP-1 may also have a role in the development of extra-cellular matrix (Langford et al. 1999). Recently, it has been shown that VAP-1 can bind soluble elastin and that elastin can inhibit VAP-1 enzymatic activity (Olivieri et al. 2010). The same research also showed that L-lysine binds to VAP-1 in the presence of H_2O_2 and acts as an uncompetitive inhibitor of VAP-1, and that L-lysine and elastin might bind to a similar site in VAP-1 (Olivieri et al. 2010).

2.1.3 Clinical relevance of VAP-1

Some reaction products of VAP-1 are cytotoxic. Methylglyoxal, for example, can form harmful advanced glycation end-products (Mathys et al. 2002). H_2O_2 produced by enzymatic reactions of VAP-1 and MAOs is a local signaling molecule that acts in an insulin-like way by stimulating glucose uptake (Enrique-Tarancón et al. 1998). When enzymatic activity of VAP-1 is increased, the excess hydrogen peroxide accumulates to a toxic concentration and contributes to formation of free radicals. It is assumed that the complications of diabetes mellitus 1 and 2 are caused by the elevated enzymatic activity of VAP-1 and its consequential H_2O_2 production (Boomsma et al. 2003, Boomsma et al. 1995, Garpenstrand et al. 1999). Therefore, VAP-1 is considered a potent target in diabetes treatment. Increased enzymatic activity of VAP-1 is also reported in chronic kidney rejection (Kurkijärvi et al. 2001), obesity (Weiss et al. 2003), and in congestive heart failure (Boomsma et al. 1997).

Altered expression levels of VAP-1 also cause diseases. Overexpression is reported in psoriasis (Madej et al. 2007), atopic eczema (Madej et al. 2006), Alzheimer's disease (Jiang et al. 2008), myopathies (Olivé et al. 2004) and in CADASIL (cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy) (Ferrer et al. 2002). Increased levels of sVAP-1 are reported in inflammatory liver disease (Kurkijärvi et al. 1998) and in relapsing-remitting multiple sclerosis (Airas et al. 2006). Decreased level of sVAP-1 has been reported with patients taking second-generation antipsychotics for schizophrenia (Roessner et al. 2007). Decreased VAP-1 expression is rare but it has been observed in depression (Roessner et al. 2006) and cancer (melanoma (Forster-Horvath et al. 2004), myosarcoma (Jaakkola et al. 1999)).

2.1.4 Reaction mechanism of CAOs

The reaction catalyzed by CAOs can be divided into two half-reactions: 1) the reductive half-reaction, and 2) the oxidative half-reaction (Fig. 5). In the initial reductive half-reaction the substrate molecule reacts with the C5 oxygen of oxidized TPQ (TPQ_{ox}) and forms a substrate Schiff base (Mure et al. 2002). The catalytic aspartate (Asp386 in VAP-1) subsequently abstracts the C_α hydrogen from the substrate molecule which yields a product Schiff base (Wilmot et al. 1997). The cofactor TPQ is concomitantly reduced (TPQ_{red}). The hydrolysis of product Schiff base results in the aldehyde reaction product (Mure et al. 2002).

In the latter half-reaction TPQ_{red} is converted back to TPQ_{ox} with help of a copper ion coordinated to three histidines (His520, His522, and His684 in VAP-1). The copper ion facilitates the transfer of protons and electrons from TPQ_{red} to O_2 (Su & Klinman 1998). Finally, addition of water releases ammonia and hydrogen peroxide as reaction products, the C5 carbonyl oxygen is restored, and TPQ_{red} is ready to react with another substrate molecule.

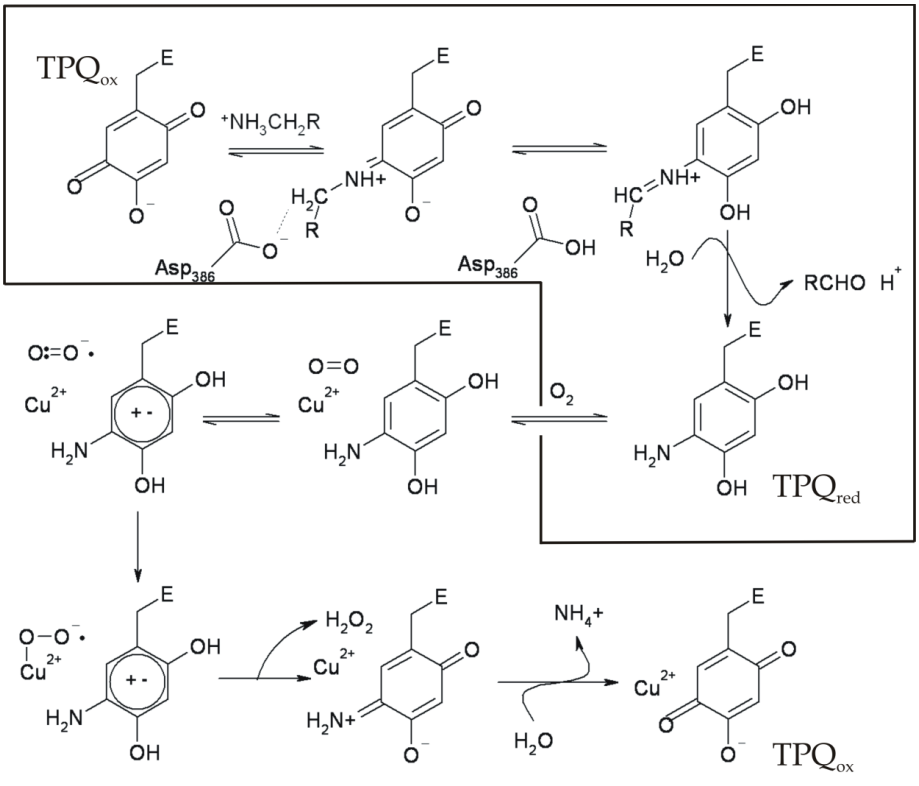


FIGURE 5 The enzymatic reaction catalyzed by TPQ. In the initial reductive half-reaction (inside the black box) TPQ_{ox} reacts with amine substrate and TPQ_{red} is formed. Addition of water releases aldehyde product. The oxidative half-reaction (outside the box) requires molecular oxygen and the copper ion. Hydrogen peroxide and ammonia are released at the end of latter half-reaction and TPQ_{ox} is reformed. Figure is modified from Mure et al. 2002.

2.1.5 Amine oxidase substrates

The FAD-containing amine oxidases and CAOs catalyze the same reaction (Fig. 2) and their substrate specificities are partly overlapping (Table 1). Some substrates were identified already decades ago but new substrates are still being found. The role of the MAOs is to degrade neurotransmitter amines. The preferred substrate for MAO A is serotonin (Ochiai et al. 2006). MAO B substrates include tyramine and 2-phenylethylamine (Ochiai et al. 2006).

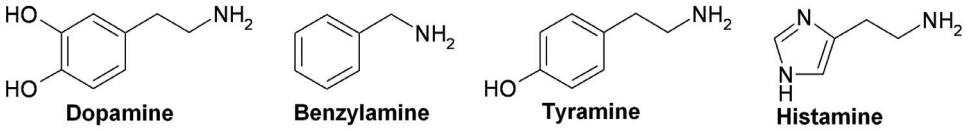


FIGURE 6 Structures of some AO substrates.

Methylamine (Lyles, 1996) and benzylamine (Fig. 6) (Dunkel et al. 2008) are substrates for VAP-1, and histamine (Fig. 6) and other diamines serve as substrates for DAO (Table 1). Little is known about RAO, a homolog of VAP-1 and DAO, but its substrate specificity has been studied (Kaitaniemi et al. 2009). The preferred substrate of RAO *in vitro* is 2-phenylethylamine. Interestingly, tyramine (*p*-tyramine, Fig. 6) is a substrate for all amine oxidases mentioned above. Dopamine (Fig. 6), 2-phenylethylamine and benzylamine, for example, can also be deaminated by more than one amine oxidase (Table 1). The specificity of amine oxidases toward amine compounds is shown to be concentrate-dependent *in vitro*, and by increasing the amine concentration the specificity is lost (Ochiai et al. 2006).

VAP-1 has physiological substrates in addition to small molecular amine substrates. It can oxidize the arginine side chain from Siglec-10 (Kivi et al. 2009), for example. L-lysine has been shown to be both an inhibitor (Olivieri et al. 2010) and a substrate (Wang et al. 1996b) of VAP-1. The fact that amine oxides can deaminate a large variety of different amine compounds complicates the design of subtype selective inhibitor molecules.

TABLE 1 Some substrates and inhibitors for AOs.

Amine oxidase	Substrate	Reference	Inhibitor	Reference
MAO A	*Dopamine*	(Li et al. 2002)	Clorgyline	(Murphy et al. 1979)
	Adrenaline	(Leeper et al. 1958)	Eugenol	(Tao et al. 2005)
	Noradrenaline	(Leeper et al. 1958)	Harmaline	(Nelson et al. 1979)
	Serotonin	(Li et al. 2002)	Brofaromine	(Da Prada et al. 1989)
	Tyramine	(Hasan et al. 1988)	Moclobemide	(Nair et al. 1993, Da Prada et al. 1989)
			Phenelzine	I
			Tranylcypromine	(Sourkes & Missala 1977)

(continues)

TABLE 1 (continues)

MAO B	*2-Phenylethylamine*	(Yang & Neff 1973)	Deprenyl (Selegiline)	(Knoll & Magyar 1972, Culpepper & Kovalick 2008)
	Benzylamine	(Newton-Vinson et al. 2000)	Isatin	(Wang & Edmondson 2007)
	Dopamine	(Paterson et al. 1995)	*Pargyline*	(Murphy et al. 1979)
	Histamine	(Ochiai et al. 2006)	Rasagiline	(Lecht et al. 2007)
			Safinamide	(Ravikumar & Sridhar 2010)
			Tranylcypromine	(Sourkes & Missala 1977)
VAP-1	*1-Methylhistamine*	(Ochiai et al. 2006)	4-Aminomethyl-pyridines	(Haider et al. 2010)
	2-Phenylethylamine	(Lyles 1996)	Carboxamides	(Clauzel et al. 2007)
	Agmatine	(Holt et al. 2003)	Hydroxylamine	(Vidrio & Medina 2007)
	Aminoacetone	(Lyles & Chalmers 1995)	*Isoniazid*	(Vidrio et al. 2000)
	Benzylamine	(Dunkel et al. 2008)	L-lysine	(Olivieri et al. 2010)
	L-lysine	(Wang et al. 1996b)	*Phenelzine*	I
	Methylamine	(Lyles 1996)	Phenylallyl-hydrazines	(Wang et al. 2006)
	Serotonin	(Ochiai et al. 2006)	*Semicarbazide*	(Ochiai et al. 2006)
	Siglec-10	(Kivi et al. 2009)	Sulfonamides	(Olarte et al. 2006)
	Tyramine	(Ochiai et al. 2006)	Thiocarbamoyl derivatives	(Yabanoglu et al. 2007)
			Tranylcypromine	(Shepard et al. 2003)
			β-aminopropio-nitrile	(Lyles & Singh 1985)
DAO	*1-Methylhistamine*	(Elmore et al. 2002)	Amiloride	(Federico et al. 1997)
	Agmatine	(Holt & Baker 1995)	Aminoguanidine	(Sourkes & Missala 1977)
	Cadaverine	(Elmore et al. 2002)	Berenil	(McGrath et al. 2009)
	Dopamine	(Yu 1988)	Clonidine	(Federico et al. 1997)
	Histamine	(Elmore et al. 2002)	Diethyldithio-carbamate	(Bardsley et al. 1974)
	Putrescine	(Elmore et al. 2002)	Iproniazid	(Burkard et al. 1960)
	Spermidine	(Elmore et al. 2002)	*Isoniazid*	(Burkard et al. 1960)
	Tryptamine	(Reed 1965)	*Pargyline*	(Sourkes & Missala 1977)
	Tyramine	(Coleman et al. 1991)	Pentamidine	(McGrath et al. 2009)
			Phenelzine	(Holt & Baker 1995)
			Semicarbazide	(Ochiai et al. 2006b)
			Tranylcypromine	(Shepard et al. 2003)
RAO	*2-Phenylethylamine*	(Kaitaniemi et al. 2009)		
	Benzylamine	(Kaitaniemi et al. 2009)		
	Tyramine	(Kaitaniemi et al. 2009)		

Compounds written in cursive bind to more than one AO

2.1.6 Amine oxidase inhibitiors

Traditionally MAO inhibitors (MAOIs) have been used to treat depression and other disordes, such as panic attacks and dementia (Riederer et al. 2004, for review, see Yamada & Yasuhara 2004). The inhibition of MAOs increases the concentration of neurotransmitters. The MAOI treatments are somewhat controversial, as oral administration of certain MAOIs can lead to hypertensive crisis (Blackwell 1963, Brown & Bryant 1988).

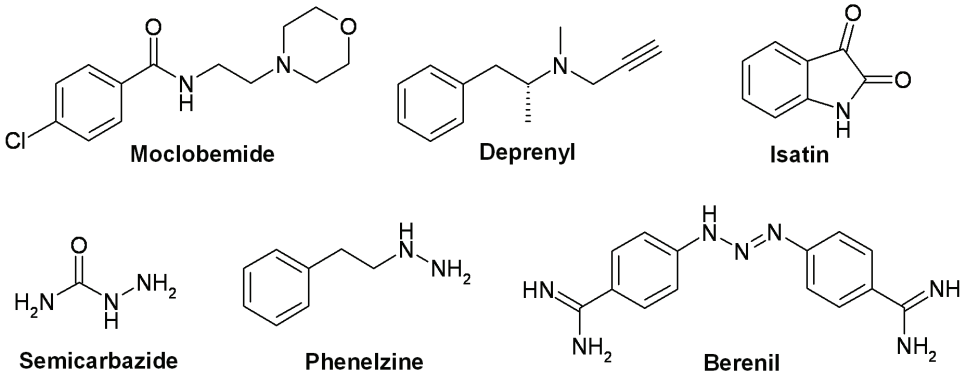


FIGURE 7 Structures of some AO inhibitors. Moclobemide, Deprenyl and Isatin are primarily MAOIs. Semicarbazide, Phenelzine and Berenil inhibit CAOs.

MAO A can be inhibited by clorgyline, harmaline and moclobemide (Fig. 7), for example. MAO B inhibitors include safinamide, pargyline and deprenyl (selegiline) (Fig. 7). In 2006 The Food and Drug Administration (FDA) approved Emsam (active substance: deprenyl) as the first transdermal patch for the treatment of depression. When administered through the skin, deprenyl does not cause the usual side effects of MAOI (Lee & Chen 2007).

Hydrazines have been used clinically to treat depression (e.g. phenelzine, Fig. 7). They are non-selective and irreversible MAOIs and they can also inhibit CAOs such as VAP-1 and DAO (Lizcano et al. 1996). The binding of a hydrazine compound 2-hydrazinopyridine (2HP) has been studied thoroughly in *Escherichia coli* amine oxidase (ECAO) (Mure et al. 2005a, Mure et al. 2005b). ECAO is very similar in structure and function to human VAP-1 and many results acquired from ECAOs, for example the studies concerning TPQ reaction mechanism, can be applied in VAP-1 studies. VAP-1 has also been crystallized in a complex with 2HP (Jakobsson et al. 2005)

Hydrazines are very reactive and they readily form a substrate Schiff base mimic with VAP-1. They are suicide inhibitors of amine oxidases (Lizcano et al. 1996), and once they have reacted with cofactor TPQ, neither the compound nor any of the reaction products can be released from the catalytic site.

2.1.7 Structures of CAOs

The structure of VAP-1 was solved in 2005 by two separate research groups (Jakobsson et al. 2005, Airenne et al. 2005) (Table 2). VAP-1 is a glycoprotein and its glycosylation sites have been predicted. In the crystal structure of VAP-1 (PDB code: 1US1) clear electron densities were found around Asn137 and Asn232 and N-acetylglucosamine sugar units were built inside them. Also, some density was detected around other potential N-glycosylation sites (e.g. Asn592) (Airenne et al. 2005) (Table 2).

TABLE 2 Summary of the available VAP-1 structures

PDB-code	1US1	1PU4	2C10	2C11
Resolution (Å)	2.90	3.20	2.50	2.90
Expression cell line	CHO[1]	CHO[1]	HEK293[2]	HEK293[2]
Amino acids visible in structure	55-201, 205-761	55-201, 205-761	41, 58-762	58-729
Inhibitors	-	-	-	2HP
TPQ	On-copper	On-copper	Off-copper	Off-copper
Glycosylation sites	Asn137[3] Asn323[3]	Asn137[3] Asn323[3]	Asn137[4] Asn323[3] Asn592[5]	Asn137[3] Asn294[3] Asn323[3] Asn592[6]
Ions	1 x Cu^{2+} 2 x Ca^{2+}	1 x Cu^{2+} 2 x Ca^{2+}	1 x Cu^{2+} 2 x Ca^{2+} 2 x Cl^{+}	7 x Cu^{2+} 2 x Ca^{2+} 2 x Cl^{+}
Disulfides (Cys-Cys)	198-199 404-430 734-741 748-748[7]	198-199 404-430 734-741 748-748[7]	41-748 198-199 404-430 734-741	198-199 404-430
Reference	Airenne et al. 2005		Jakobson et al. 2005	

[1] Chinese hamster ovary cells
[2] Human embryonic kidney cells
[3] NAG = N-acetyl-D-Glucosamine
[4] NAG, BMA = β-D-Mannose
[5] NDG = 2-(acetylamino)-2-deoxy-A-D-Glucopyranose, NAG, FUL = β-L-Fucose
[6] NAG
[7] Inter-subunit disulfide bond

The biologically active unit of VAP-1 is a heart-shaped homodimer (Fig. 8) that is attached to the cell membrane *via* a transmembral anchor. Each VAP-1 subunit has two long arm-like protrusions that extend around the other subunit.

One of the protrusions is a structural part of another subunit's active site (Fig. 8). A disulfide bond between the adjacent cysteins 198 and 199 of each monomer contributes to the stability of the dimer (Airenne et al. 2005) and may have other roles as well (Olivieri et al. 2010). Other disulfides are also present (Table 2).

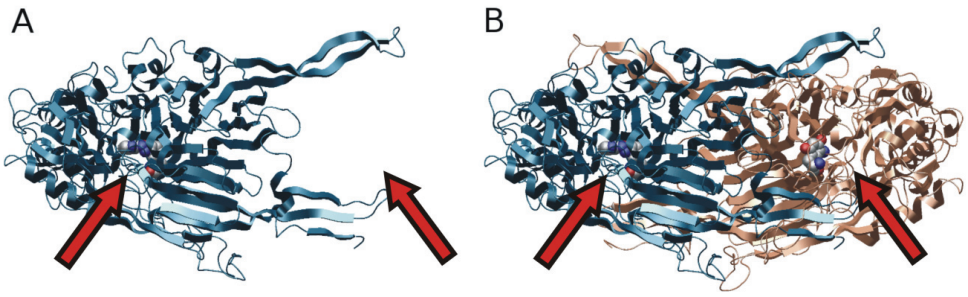


FIGURE 8 The 3D structure of VAP-1. The TPQ-2HP complexes are shown in van der Waals spheres. A. The structure of a VAP-1 monomer, and the orientation of the arm-like protrusions. The protrusion from one monomer is a structural part of the cavity which leads to the active site of another monomer (arrow). B. The structure of a biologically active dimer. Red arrows indicate the cavities that lead into the active site.

The available 3D structures show that TPQ has two possible conformations: 1) the on-copper conformation (the inactive conformation), or 2) the off-copper conformation (the active conformation) (Fig. 9). In the inactive conformation the C5 oxygen is directly coordinated to the copper ion. In the active conformation the C2 oxygen forms a hydrogen bond to a water molecule that coordinates to the copper ion, and the C5 oxygen is available at the active site and locates near the catalytic Asp386.

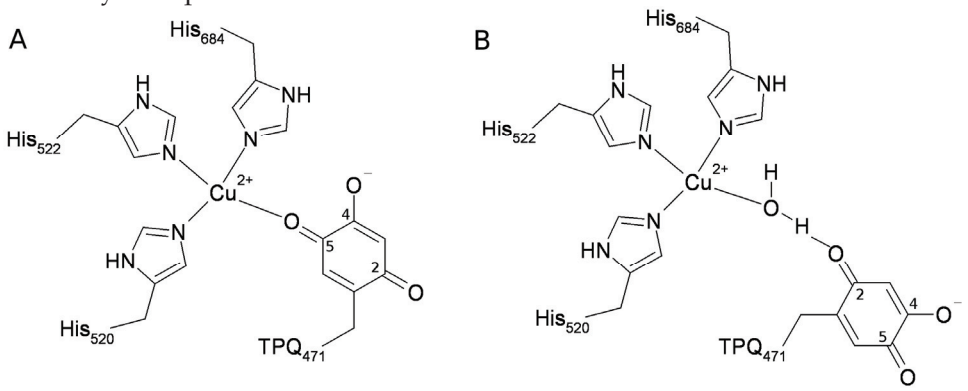


FIGURE 9 Schematic representations of the two possible conformations of the TPQ. A. The inactive conformation of TPQ where the reactive C5 oxygen is coordinated to the copper ion. B. The active conformation of TPQ where the C5 oxygen is available at the active site and the TPQ is bonded to the copper ion indirectly, *via* a water molecule.

The active site of VAP-1 is located at the center of a monomer, which is the most conserved area throughout CAOs (Airenne et al. 2005). The conserved amino acids include: 1) Tyr471, which constitutes the cofactor TPQ, 2) the catalytically active base Asp386, 3) Asn470 and Tyr372 that contribute to the correct orientation of the TPQ, and 4) His520, His522, and His684 that are coordinated to the copper ion (Airenne et al. 2005). The active site of VAP-1 is mainly hydrophobic, although many hydrogen bond donors and acceptors are present, for example, hydroxyl groups of tyrosine residues and the catalytic Asp386. The cofactor TPQ is located at the far end of the narrow ligand-binding pocket.

The structure of DAO was recently published (McGrath et al. 2009) and the folding of VAP-1 and DAO is very similar even though their sequence identity is a modest 40 %. The 3D structures enable rational ligand discovery since the amino acid differences can be compared between different CAOs. Currently, there is no structure available for RAO (68 % sequence identity to VAP-1) but homology modeling suggests that the shape of the ligand-binding pocket of RAO is similar to that of VAP-1's (Kaitaniemi et al. 2009).

Few amino acid differences can be found inside the active sites that impact on the substrate specificities of DAO and VAP-1 (McGrath et al. 2009). A specific feature of DAO is that, in addition to the catalytic aspartate, another aspartate (Asp186) is located in the active site cavity. In VAP-1 the corresponding amino acid is Thr212. The crystal structures of DAO in complex with non-covalent inhibitors berenil (PDB code: 3HIG) and pentamidine (PDB code: 3HII) reveal the important role of Asp186 in substrate specificity, as Asp186 can stabilize the diamine substrate compound from one end while the other end reacts with TPQ (McGrath et al. 2009).

The entry of substrates into the VAP-1 ligand-binding pocket is regulated by Leu469 which can either allow ligands to enter, or block the access to the active site, depending on its conformation (Airenne et al. 2005). In DAO the corresponding amino acid is Tyr459 which is a bulkier amino acid than leucine in VAP-1 and most likely is unable to undergo a similar conformational fluctuation.

2.2 Filamins

Filamins (FLNs) are cytoskeletal proteins that participate in the formation of orthogonal F-actin networks. Three isoforms of FLNs, A, B, and C are present in humans; these share a significant sequence identity (70 %). FLNc is mainly expressed in striated muscles, whereas FLNa and FLNb are expressed throughout the body (Stossel et al. 2001). Filamin orthologs are found in G*allus gallus, Drosophila melanogaster, Caenorhabaditis elegans, Dictyostelium discoideum* and *Entamoeba histolytica*.

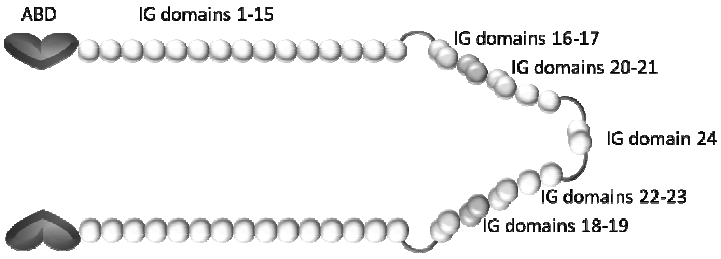


FIGURE 10 The organization of FLNa dimer. Actin binding domain (ABD) is located at the N-terminal end of polypeptide chain followed by 15 individual Ig-like domains and a linker before domain pairs 16-17, 18-19 and 20-21. Domains 22 and 23 precede another linker and in C-terminal end the domain 24 is responsible for the dimerization of FLNa.

FLNa is a V-shaped, parallel homodimer (Fig. 10). Each 280 kDa subunit consists of an N-terminal actin binding domain and 24 repeating immunoglobulin-like (Ig-like) domains (Fig. 10) of which the most C-terminal domain mediates the dimerization (Pudas et al. 2005). The Ig-like domain structure is a β barrel of seven β strands (A–G) that from two antiparallel β sheets (Fig. 11). FLNa participates in numerous interactions with other proteins, actin being the most abundant.

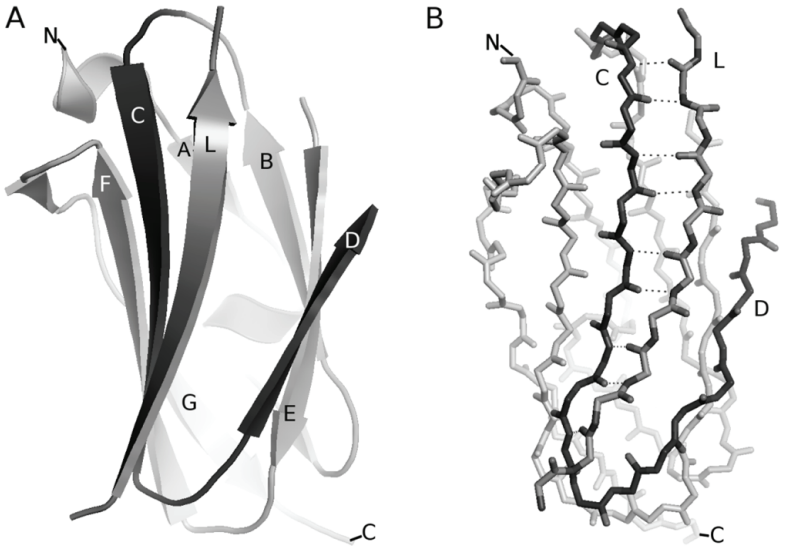


FIGURE 11 The structure of the FLNa Ig-like domain. A. The Ig-like domain is a β barrel of two antiparallel β sheets. One β sheet is formed by A, B, D, and E strands, another by C, F, G, and L (ligand) strands. Ligands bind between strands C and D. B. Ligand forms an additional β strand next to the C strand (side chains are omitted). The binding occurs *via* main chain hydrogen bonds (dashed lines). N- and C-terminals are marked with N and C letters, respectively. Figures are generated n the basis of the structure of FLNa21 in complex with β2 integrin peptide (PDB code: 2JF1, Takala et al. 2008).

Different Ig-like domains of FLNa bind distinct proteins, for example, the cytosolic tails of integrin β subunits (Kiema et al. 2006, Takala et al. 2008), migfilin (Lad et al. 2008), and cystic fibrosis transmembrane conductance regulator (CFTR) (Smith et al. 2010). Multiple crystal structures and NMR-structures of FLNa domains in complex with peptides, derived from the directly interacting counterpart proteins, have been solved and are available in the Protein Data Bank (PDB) (Berman et al. 2000). The binding of many FLNa interaction partners occurs between the β strands C and D (CD-face) (Fig. 11). CD-faces are similar in sequence between Ig-like domains, yet some differences are also present, such as the amino acid composition and sequence length between the C and D strands, which determines the selectivity of a certain Ig-like domain for a specific target protein. Domains 9, 17, 19, 21, and 23 have more similarity to each other, than to the rest of the Ig-like domains.

The physiological role of FLNa is to act as a mechanosensor. The Ig-like domains 1 to 15 are each most likely individual domains whereas domains 16-21 form dimers: 1) domains 16-17 (Heikkinen et al. 2009), 2) domains 18-19 (Heikkinen et al. 2009), and 3) domains 20-21 (Lad et al. 2007). In the relaxed state of the actin network these dimers stay intact and the CD-faces of the odd-numbered domains are masked by the A strands from the preceding domains, for example the CD-face of domain 21 is masked by the A strand of domain 20 (Lad et al. 2007). The binding of the A strand from the previous domain is similar to the binding of natural ligand proteins (Fig. 11): the A strand forms an additional β strand next to the C-strand.

It has been hypothesized that when the actin network begins to move, FLN detects the movement, the domain complexes are pulled apart, and the masked CD-faces are revealed. The availability of a certain domain binding site would thus be regulated by the stress level of the actin network. (Pentikäinen & Ylänne 2009)

2.2.1 Cystic fibrosis transmembrane conductance regulator

Cystic fibrosis is a hereditary autoimmune disease that is most common among Caucasians. The symptoms include a high chloride concentration of sweat, accumulation of thick mucus inside the lungs, and respiratory infections. Most cases (~70 %) of cystic fibrosis are caused by a ΔF508 mutation in a CFTR protein, which is an apical chloride channel of epithelial cells. The ΔF508 mutation causes abnormally folded CFTR proteins that are unable to reach their destination, the plasma membrane, but are retained in the ER instead (Thibodeau et al. 2010). The ΔF508 mutation is an appealing target for the pharmaceutical industry, since the abnormally folded proteins do posses some ion channel activity (Denning et al. 1992) even though their residency at the plasma membrane is short lived. Other cases of cystic fibrosis are usually caused by a variety of mis-sense mutations of CFTR, e.g. S13F.

The N-terminus of CFTR is a vital part of the protein and it has many roles: 1) It is needed for the biosynthesis of the normal protein, since truncation of 80 amino acids at the N-terminus disturbs the protein folding (Prince et al. 1999), 2)

the N-terminus of CFTR interacts with chaperones that aid CFTR to fold correctly (Zhang et al. 2002, Zhang et al. 2006), 3) amino acids 29-31 form an ER retention motif (Chang et al. 1999), and 4) the interaction between the N-terminus and the regulatory domain (R domain) of CFTR is needed for autoregulation of chloride channel gating (Naren et al. 1999), as disruption of the interaction decreases channel gating. In addition to autoregulation, cAMP dependent kinases (protein kinase A) also regulate CFTR by phosphorylating the protein in multiple locations (Chang et al. 2002, Chappe et al. 2005, Mense et al. 2006). Other regulators of CFTR are soluble N-ethyl maleimide–sensitive factor attachment protein receptors (SNAREs), syntaxin 1A, and SNAP23 (Cormet-Boyaka et al. 2002, Naren et al. 1997).

2.2.2 The interaction between filamins and CFTR

Recently, FLNa and FLNb have been identified as direct binding partners of the most N-terminal part of CFTR (Thelin et al. 2007). The inhibition of the actin assembly decreases the cell surface density of CFTR (Cantiello, 1996). Accordingly, the interaction with FLNs is needed for the stable CFTR organization at the cell surface. As a variety of *in vitro* and *in vivo* experiments confirm, FLNs have a role in cell surface stability and they regulate the spatial organization of CFTR by coupling the channel to the actin cytoskeleton (Thelin et al. 2007). The S13F mutation of CFTR results in poorer stability and a fivefold decreased concentration of S13F CFTR in the plasma membrane, compared to the wild type (WT) CFTR. However, most of the S13F mutant CFTR proteins do not ever reach the plasma membrane but end up in lysosomes instead (Thelin et al. 2007).

The structure of FLNa21 in complex with CFTR S13F peptide (Smith et al. 2010) was published in the same issue of the Journal of Biological Chemistry in tandem with the article III. Smith et al. have explained the structural basis of the effects of S13F mutation to the CFTR binding with FLNa. The same results were acquired in article III independently by combining molecular modeling with experimental methods.

2.3 T-cell protein tyrosine phosphatase, a novel anti-cancer target

Phosphorylation of proteins is an important regulatory process in eukaryotic and prokaryotic organisms. Phosphorylation occurs to specific amino acids; in the eukaryotes phosphorylation involves mainly serines, threonines, and tyrosines. Phosphorylation is mediated by enzymes, called kinases, for example, tyrosine phosphorylation is mediated by protein tyrosine kinases. Many cellular pathways are regulated by reversible phosphorylation.

In order to control the cellular pathways, another family of enzymes called phosphatases, is needed. They remove phosphate groups from the phosphorylated proteins and thus, counteract the role of kinases. Protein

tyrosine phosphatases (PTPs, EC 3.1.3.48) constitute a family of proteins that remove phosphate groups from phosphotyrosines. There are 107 genes in human genome that encode the members of this family (Alonso et al. 2004).

T-cell protein tyrosine phosphatase (TCPTP; also known as PTPN2) belongs to a subfamily of intracellular non-receptor PTPs. Two isoforms of TCPTP are present in humans: 1) the nuclear 45 kDa form (TC45), and 2) the endoplasmic reticular 48 kDa (TC48) form (Cool et al. 1989). TCPTP was discovered in the late 1980s (Cool et al. 1989), shortly after a closely related protein, PTP1B, was characterized (Tonks et al. 1988a) and purified (Tonks et al. 1988b).

Endothelial growth factor receptor (EGFR) is a receptor tyrosine kinase (RTK) and it is able to activate itself *via* autophosphorylation in response to the endothelial growth factor binding to the receptor. The activation of EGFR induces TC45 translocation from the nucleus to the cytoplasm (Tiganis et al. 1998). The cytoplasmic TC45 is activated by the α_1 cytoplasmic tail of $\alpha_1\beta_1$ integrin and the activated TC45 dephosphorylates EGFR, thus regulating it negatively (Mattila et al. 2005) (Fig. 12).

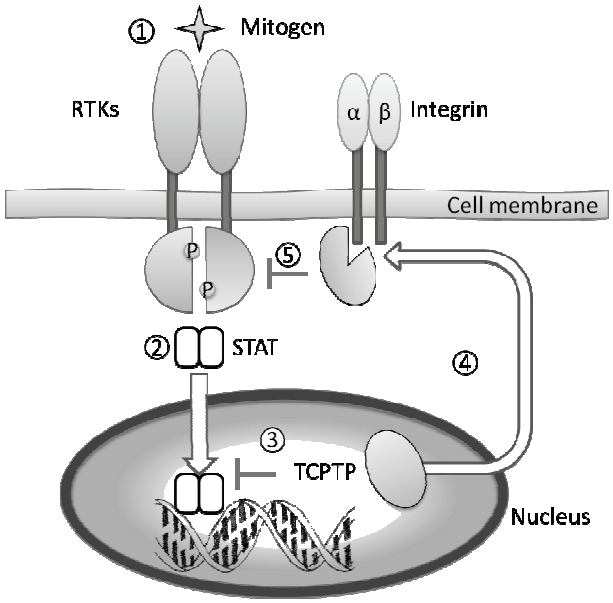
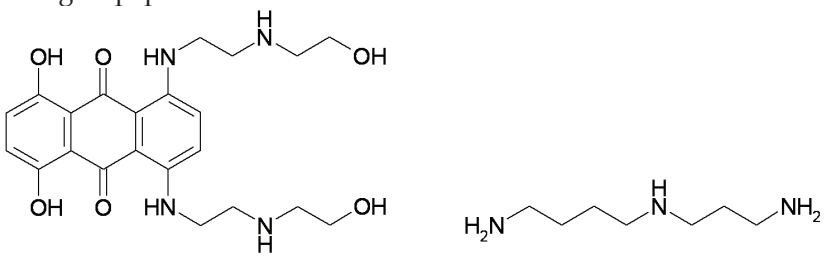


FIGURE 12 TCPTP controls cell cycle negatively. 1. Mitogens bind to the receptor tyrosine kinases (RTK) which triggers the (auto-) phosphorylation of RTKs. 2. Phosphorylated RTKs activate STAT signal transduction cascade. 3. Activated STAT-complex binds to DNA and nuclear TCPTP is able to inactivate STAT. 4. RTK activation stimulates TCPTP translocation to cytoplasm. 5. TCPTP is activated by α_1 integrin tail and activated TCPTP can dephosphorylate RTKs.

TC45 also regulates the vascular endothelial growth factor receptor 2 (VEGFR2) (also an RTK) and, as a result of TCPTP binding to VEGFR2, VEGFR2 signaling, and the growth, migration and differentiation of human endothelial cells are inhibited (Mattila et al. 2008). TC45 has a role in negative regulation of cell cycle as it directly dephosphorylates for example Janus kinase 1, and Signal transducer and activator of transcription (STAT) 3 (Shields et al. 2008), STAT-1 (ten Hoeve et al. 2002), and STAT-5 (Aoki & Matsuda 2002) (Fig. 12).

TCPTP is composed of a catalytically active domain and a C-terminal tail domain. The structure of catalytic domain of TCPTP was solved in 2002 (Iversen et al. 2002). The domain fold is conserved throughout the family of non-receptor PTPs and they all contain a catalytically active cystein. The sequence identity between the catalytic domains of TCPTP and closely related protein PTP1B is 74 %. The C-terminal tail of TC45 has been reported to function as an auto-inhibitory domain (Hao et al. 1997). The removal of an 11 kDa segment from the C-terminus of WT TCPTP resulted in elevated enzymatic activity of TCPTP (Zander et al. 1993). To date there is no structural information about the auto-inhibitory C-terminal domain, as the structure of TCPTP only contains amino acids 4-277 (Iversen et al. 2002) of total 387 amino acids of TC45.

Recently, six activators of TCPTP have been discovered (Mattila et al. 2010). Two of these compounds (mitoxantrone and spermidine, Fig. 13) were shown to be TC45 specific. Mitoxantrone was shown to compete with α_1 integrin peptide, which suggests that the binding of mitoxantrone is similar to that of integrin peptide.



Mitoxantrone **Spermidine**

FIGURE 13 Structures of TCPTP activators mitoxantrone and spermidine.

TCPTP is an appealing target for cancer treatments, as it negatively regulates many receptor tyrosine kinases relevant to cancer (Mattila et al. 2005, Mattila et al. 2008). In addition, TCPTP is a good tumor suppressor, as its expression is not turned off in cancers (Lu et al. 2007). Mitoxantrone is an immunesuppressant and it is currently used in the treatment of breast cancer (Yap et al. 1981), relapsed acute lymphoblastic leukaemia (Parker et al. 2010), and multiple sclerosis (Rizvi et al. 2004, Stûve et al. 2004). It is also known to interfere with both DNA and RNA, and it may inhibit topoisomerase II (Fox, 2004).

Spermidine, instead, is a biogenic polyamine able to bind DNA (Rubin, 1977) and proteins, for example, PAO. Neither of the compounds is suitable for use as a therapeutic TCPTP activator because of their unspecificity and side-effects, but they are valuable compounds for the study of the TCPTP function.

3 AIMS OF THE STUDY

I The aim was to understand the structural and functional differences between amine oxidases. This allows the development of subtype-selective molecules, which in turn, can be used as tools to identify the role of different proteins in diseases, and maybe even as lead compounds in the development of therapeutics against those diseases.

II The aim was to investigate the structural basis of Filamin A subdomain selectivity toward CFTR. The collaborators had experimental results and our aim was to explain their results by using molecular modeling.

III The collaborators had recently identified six novel activators for anti-cancer target TCPTP. Our aim was to use those molecules as tools to identify the activator binding site in TCPTP and also to characterize the binding experimentally.

4 METHODS

The methods used in this thesis are summarized in the table below (Table 3). Detailed descriptions are found in the publications indicated by Roman numerals. The relation of methods used in this thesis to the whole rational drug discovery procedure can be seen in Fig. 1.

TABLE 3 Summary of methods.

Method	Publication
Molecule sketching and minimization	I, II, IV
3D QSAR	I
Molecular docking	I, II, IV
Molecular dynamics (MD)	I, II
Structure-based sequence alignment	III
Protein expression and purification	IV
Differential scanning fluorimetry (DSF)	IV
Isothermal titration calorimetry (ITC)	IV

5 RESULTS

5.1 VAP-1 and hydrazines

5.1.1 3D QSAR model based on novel hydrazines (I)

Hydrazines are well known inhibitors of VAP-1. In this study, 46 novel hydrazine molecules (I, Table 1) were developed and used with the commercially available compound phenelzine to construct three-dimensional quantitative structure-activity relationship (3D QSAR) models for VAP-1 and MAO.

The experimental data for the hydrazines, IC_{50} values measured for VAP-1 and total MAO, show that the molecules inhibit AOs. All compounds in this molecule set are larger than the 2HP crystallized in complex with VAP-1 (Jakobsson et al. 2005). The flexibility and dynamics of the ligand-binding pocket of VAP-1 was therefore studied by performing MD simulations with **5a**, which was chosen because of its simple structure and strong selectivity toward VAP-1 over total MAO. In the course of simulation, the Leu469 side chain turned from the "closed" conformation seen in the crystal structures (Jakobsson et al. 2005, Airenne et al. 2005) to an open conformation, leaving the cavity leading to the ligand-binding pocket of VAP-1 open (I, Fig. 2 D). Met211 and Ser496 also turned and at the end of the simulation extra space was clearly visible behind Met211 (I, Fig. 2 D). This suggests that molecules far larger than 2HP, and the others in this molecule set could bind inside the VAP-1 ligand-binding pocket.

A simple structure-activity relationship study based on the super-positioning of molecules is not sufficient to understand the factors contributing to VAP-1 activity or the selectivity over MAO. Therefore, the 3D QSAR technique was employed to build models that explain the binding of these hydrazines in more detail. Instead of an alignment of molecules on top of each other, the superpositioning was acquired by docking the molecules inside the active site of the VAP-1 crystal structure, where the conformation of Leu469

was changed to resemble the conformation seen in the MD simulations (I, Fig. 2 D). This way the models were more relevant, as the bioactive conformations of molecules were employed.

The comparative molecular similarity indices analysis (CoMSIA) models were built for VAP-1 and MAO by using the leave-one-out (LOO) method. The docking of hydrazines inside MAO ligand-binding pocket was difficult. Thus, the superpositioning of molecules acquired from the VAP-1 docking was used also for the building of MAO models. The shape of the MAO ligand-binding pocket allows molecules to be docked in many different conformations, which results in poor superpositioning of molecules. Also, the MAO reaction mechanism with hydrazines is problematic. It is probable, that the hydrazine moiety of the molecules is cleaved off during the reaction (Binda et al. 2008). This would have caused perturbation inside the data set, as the molecules **phenelzine, 3a, 3n,** and **3q** all would have resulted in similar structures after deletion of the hydrazine moiety, even though they all have distinct IC_{50} values.

The parameters for the model construction are shown in article I, Table 2. The final model for VAP-1 was acquired by using 4 partial least squares (PLS) components. The correlation coefficients q^2_{LOO}: 0.636 and r^2: 0.828 were obtained by means of a steric CoMSIA field for the VAP-1 model. The best correlation for MAO was found by using a hydrophobic CoMSIA field, which gave correlation coefficients of q^2_{LOO}: 0.749 and r^2: 0.840 with 3 PLS components.

The validation of the model building employed three approaches. First, the dataset was divided into four equally diverse subsets according to the IC_{50} values of VAP-1, of which three were used to train the model, and the fourth served as a test set. For both VAP-1 and MAO four submodels were built and the statistics are shown in article I, Table 2. Secondly, a progressive scrambling method (Clark & Fox 2004) was used. This method tests the sturdiness of the model by computationally adding errors to the data set. Thirdly, the bootstrapping method (Henderson 2005) was employed for further validation. The statistics show that the final models were robust and reliable (I, Table 2).

Because the molecules were initially docked to the VAP-1 structure the steric CoMSIA fields could be visualized inside it (I, Fig. 4 C). The interpretation of fields was thus more meaningful than the interpretation of fields without the structural information of the protein. The visualization of the MAO CoMSIA fields with the VAP-1 fields suggested that the areas near the hydrazine part of the molecules should not contain large substituents, since substituents larger than hydrogen are disadvantageous for the inhibitory effect toward both VAP-1 and MAO (I, Fig. 4C). The same trend can be seen when comparing the logarithmic IC_{50} values (pIC_{50}) in molar concentration, of molecules with increasing sizes of the R1 substituent (I, Table 1), 1) **phenelzine, 3a, 3n,** and **3q** (pIC_{50} M = 7.70, 6.96, 6.73, and 6.36 respectively) or 2) **5a, 9a**, and **9j** (pIC_{50} M = 7.19, 6.68, and 6.46, respectively). The selectivity toward VAP-1 over total MAO decreases as soon as the R1 substituent size exceeds that of a methyl group (I, Table 1). The most selective compounds in this series are the ones which contain a hydroxyl group in position R2 (I, Table 1). Addition of substituents to R3 or

R4 negatively affects the selectivity, especially if both positions are substituted (e.g. **10d**).

A large favorable steric field is located in the passage leading to the active site (I, Fig. 4 C). According to the crystal structure of VAP-1 there is a lot of space for a ligand to accommodate. The molecules that possess *para* substituents in position R (I, Table 1) bind to VAP-1 better than corresponding compounds without *para* substitution. For example, the addition of *p*-methoxy to **3a** and **5a** results in the more potent **3i** and **5c**, respectively (I, Table 1). Two unfavorable steric fields are located between R and R2. The comparison of **9a** and **9i** shows that the VAP-1 potency is decreased when phenyl is replaced by naphthyl (I, Table 1).

The MAO fields are more difficult to interpret; however, they can be used to guide the synthesis of new VAP-1 inhibitors. The addition of hydrophobic substituents should be avoided in the areas that are predicted to increase MAO potency. It can also be seen from Table 1 in article I, that the compounds which have hydrophobic methyl groups in R2 (**12**) are less selective toward MAO than the molecules which have hydrophilic hydroxyl groups in R2 (**9**).

The final 3D QSAR models were used to predict the potencies of 5 new hydrazine molecules (I, Table 3). All the molecules were predicted with reasonable accuracy, taking into account that **15** and **16** were significantly different than any of the molecules used for the 3D QSAR model building. **15** was also shown to be extremely selective toward VAP-1 over MAO (I, Table 3).

The 3D QSAR models introduced in I are the first available for VAP-1. They can be used to guide the synthesis of more selective covalent or non-covalent VAP-1 inhibitors. However, if models are used to design non-covalent inhibitors, the area near TPQ should be examined cautiously, as the conformation of TPQ might be either active or inactive.

5.1.2 Dynamics of VAP-1 ligand-binding pocket (II)

The results in I suggested that the VAP-1 ligand-binding pocket might be able to accommodate larger molecules than those developed (I). A set of 8 molecules (II, Table 1) was designed according to the 3D QSAR models constructed previously (I), and their binding inside the ligand-binding pocket of VAP-1 was modeled.

The smallest molecules of the set, **2a** and **b** (II, Table 1) were docked inside the VAP-1 structure, and the conformation of Leu469 was changed to resemble the "open" conformation previously discovered (I). The MD simulations with VAP-1 in complex with **2a** and **2b** indicate that the surrounding amino acids Tyr372, Tyr384, Tyr394, Leu468, and Leu469 form a hydrophobic pocket. Both **2a** and **2b** were able to form tight hydrogen bond networks *via* their hydroxyl groups with the catalytic base Asp386 and the phenyl rings of both molecules fitted nicely inside the hydrophobic pocket (II, Fig. 3). The difference in VAP-1 potency between **2a** (IC_{50} = 0.04 μM) and **b** (IC_{50} = 0.15 μM) is probably caused by the differing orientation of the hydroxyl group (**2a** = 1*R*, **2b** =1*S*). The hydroxyl group of **2a** has plenty of space whereas the hydroxyl group of **2b** has

less space and it points toward the aromatic Tyr384 which is an unfavorable interaction, and accordingly, explains the difference in VAP-1 potency between **2a** and **2b**.

The MD simulations of VAP-1 with hydrazine inhibitors **2a** and **2b** suggest that the VAP-1 ligand-binding pocket is able to expand compared to the size seen in the crystal structure (Airenne et al. 2005). In the course of the simulations the conformational change of Met211 revealed extra space. In DAO the corresponding amino acid is Thr185 (McGrath et al. 2009), which is a very stiff amino acid and certainly not able to adopt the versatile conformations of methionine. Moreover, opposite to Met211 is Ser469 in VAP-1, which is a quite small amino acid, in contrast to the larger His486 in DAO. Taken this together, it is likely, that the DAO ligand-binding pocket is unable to expand in the same way as the VAP-1 ligand-binding pocket; a very interesting detail that needs to be taken into consideration when new VAP-1 selective pharmaceuticals are designed.

The binding of larger hydrazine compounds was studied by docking the molecule **8** (II, Table 1) into the VAP-1 structure acquired from the MD simulation of VAP-1 with **2a**. Molecule **8** forms a similar hydrogen bond network as **2a** and **2b**. The propyl group in position C2 extends toward the extra space created by the rotation of Met211, but does not quite reach the extra space, because of the tight hydrogen bond network (II, Fig. 4 A).

According to the docking simulations of **8**, substituents larger than propyl might be able to fit inside VAP-1. **11a–d** have phenyl substituent in C2 (II, Table 1). The binding of the different isomers of **11** was difficult to predict by using a static protein; therefore, the MD simulations were performed for **11a–d**. In the cases of all isomers, Met211 was turned in the course of simulation, like in the MD simulations of VAP-1 with **2a** and **2b** (II, Fig. 5A-D). Surprisingly, two distinct hydrogen bond networks were detected: 1) in simulations of VAP-1 with **11a–b** the C1 hydroxyl group formed a hydrogen bond with TPQ (II, Fig. 5A-B); and 2) in simulations of VAP-1 with **11c–d** the C1 hydroxyl group formed similar hydrogen bond network with Asp386, as was seen in the simulations of VAP-1 with **2a** and **2b** (II, Fig. 5C-D). In simulations with **11c** and **11d** Leu469 returned to the conformation, that blocks the entrance to the ligand-binding pocket, also seen in the crystal structure (Jakobsson et al. 2005, Airenne et al. 2005), whereas in simulations with **11a** and **11b** Leu469 remains in the conformation, that allows ligands to enter the ligand-binding pocket.

In the molecule set in II, the effect of N-methylation is positive on the VAP-1 potency, in contrast to the results acquired from the previous molecule set (I). The binding of the non-methylated **8** and the N-methylated **12** (II, Table 1) was studied by docking **12** inside the VAP-1 structure obtained from the MD simulation of VAP-1 with **2a**, and by comparing the best conformations of **12** and **8** (II, Fig. 4A-B). The binding of **12** to VAP-1 is distinct from the binding of **8,** as the addition of N-methyl abolishes the nitrogen's ability to function as a hydrogen bond donor in **12**. Thus, the hydrogen bond network is weaker in **12**, which allows the rest of the molecule to adopt a more optimal conformation,

than in the case of **8**, where the hydrogen bonds dictate the conformation of the phenyl ring. The propyl group at the C2 of **12** is able to extend further toward the extra space than was the case in **8**. This could explain why the binding to VAP-1 of **12** is 4.6 times better than that of **8** (comparison of IC_{50} values).

The general trend in this molecule set was that the smaller molecules were better inhibitors of MAO than the larger molecules, and of the larger molecules the ones without N-methyl were better inhibitors of MAO than those with N-methyl. Docking of **8** and **5** to the crystal structure of MAO-B (Binda et. al. 2008) revealed that **8** fitted inside the MAO-B ligand-binding pocket but that **5** was not able to fit inside (II, Fig. 6A-B). The negative effect of N-methylation to MAO potency might be caused by the lowered reactivity of the hydrazine moiety. Interestingly, the effect of N-methylation was opposite that in the previous set (I), meaning that in this set of molecules the N-methylation together with the addition of large C2 substituents increased selectivity toward VAP-1.

5.2 CFTR and filamin A (III)

The Ig-like domains of FLNs contain a well-characterized binding site, called the CD-face which is located between β strands C and D (Fig. 11). The domains near the C-terminal end have similar CD-faces and they share some binding partners. The binding of CFTR to FLNa was studied computationally by aligning the CD-faces of the FLNa domains based on their amino acid sequence and available 3D structures.

The alignment of domains indicated that the composition and the length of amino acids between the C and D strands have an important role in determining whether the CD-face is able to accommodate an additional β strand. Ig-like domains 4, 9, 12, 17, 19, 21, and 23 all have a similar composition of amino acids between the C and D strands (III, Fig. 5 D), as each of them contains a conserved GPS/C sequence. Domain 8 has a few extra amino acids at the beginning of the D strand which most likely contributes to a structurally different CD-face compared to other domains (III, Fig. 5 D).

In binding experiments, the WT CFTR peptide was shown to bind to domains 9, 12, 17, 19, 21, and 23 but not to domain 4, which was thought to act similarly to domains 9, 12, 17, 19, 21, and 23, according to alignment results. Structural study of the domains revealed that domain 4 has a phenylalanine in a position where all other CFTR binding domains have one of valine, leucine, or isoleucine. Phenylalanine might create steric hindrance and impede CFTR's ability to form an additional β strand in the CD-face.

A peptide containing the cystic fibrosis-causing mutation S13F was unable to bind to any of the domains in the binding experiments. The plausible explanation for this is that the S13F mutation eliminates the hydrogen bond between Ser13 in CFTR and the main chain carbonyl oxygen atom of Val or Ala (Val2472: domain 23 numbering) in domains 9, 12, 17, 19, 21, and 23. In addition,

the bulky Phe in CFTR, which replaces the Ser13 residue, cannot stack with Val or Ala (III, Fig. 5 A) or fit inside the same space as Ser.

5.3 TCPTP activation (IV)

Recently, six novel activators of TCPTP were identified, including mitoxantrone and spermidine (Mattila et al. 2010) (IV, Fig. 1). Our aim was to identify the binding site for these ligands by combining experimental and computational methods. Other known ligands of TCPTP are the C-terminal domain of TCPTP (inhibitor) and the cytoplasmic tail of α_1 integrin (activator). All these ligands contain a positive charge at physiological pH, as the modulator ligands have protonated amino groups and the protein ligands possess the positively charged amino acids lysine and arginine.

The binding of mitoxantrone and spermidine to the constitutively active form of TCPTP, TC37, was studied *in vitro* by using isothermal titration calorimetry (ITC). Results indicated that spermidine does not bind to the catalytic domain of TCPTP but mitoxantrone does (IV, Fig. 2). The binding of mitoxantrone to TC37 is endothermic, driven by entropy and occurs in a 1:1 stoichiometry.

The binding site for mitoxantrone and spermidine was not known, thus molecular modeling was utilized. On the surface of TCPTP structure (PDB code: 1L8K) (Iversen et al. 2002), near the N-terminus, is a large, hydrophobic groove which is surrounded by the negatively charged glutamate residues E8, E11, E24, and E28. This groove was selected as the probable binding site for the activators (IV, Fig. 3.) based on the knowledge that the inhibitory C-terminal domain, the activating α_1 tail and the activator molecules all possess positive charge. Bioactive conformations for activators were created by using the GOLD 3.1.1 docking program, and the best conformations were selected for the MD simulations with the WT TCPTP model structure.

The crystal structure of TCPTP (PDB code: 1L8K) (Iversen et al. 2002) only contains amino acids 5-277 of the total 387 amino acids of the TC45 isoform. The structure of a closely related protein, PTP1B (PDB code: 2VEV) (Douty et al. 2008) was used as a template and as a result, amino acids 1-4 and 278-298 were built to complement the crystal structure of TCPTP. The model, however, excluded the C-terminal domain that is responsible for controlling the activity of TCPTP *via* intramolecular auto-inhibition (Hao et al. 1997). The ITC results were supported by MD simulations of TCPTP model with spermidine and mitoxantrone, as spermidine was unable to remain bound to TCPTP during a 6 ns MD simulation. In contrast, mitoxantrone remained bound throughout the simulation (IV, Fig. 4) which indicates the stability of the mitoxantrone-TCPTP complex.

The importance of the glutamate residues in the groove to the binding of mitoxantrone was studied by performing MD simulations with double mutant TCPTPs in which the glutamate residues were mutated to alanine residues

(E8.11A and E24.28A) and by calculating the change in enthalpy, ΔH, by using the MM/GBSA method. According to the ΔH values, the WT TCPTP is able to bind mitoxantrone better than either of the double mutants (IV, Table 1). The deletion of negative amino acids did not, however, abolish the binding of mitoxantrone entirely. This is in line with the ITC measurements, as the binding of mitoxantrone is driven by entropy and the electronic interactions play a minor role in the binding.

The large entropic factor of the binding can be explained by visualizing the bound mitoxantrone inside its binding pocket (IV, Fig. 3B). The pocket is formed by hydrophobic amino acids, L12, L21, I25, I244, L248, L265, and Y269 and the hydrophobic core of mitoxantrone (IV, Fig. 1) is able to mask them during binding. The hydrophilic arms of mitoxantrone extend to the opposite sides of the groove occupied by the core of mitoxantrone. Both arms form hydrogen bonds with the glutamates (IV, Fig. 3B).

To further verify the suggested interactions between the flexible arms of mitoxantrone and the glutamates of TCPTP, two mitoxantrone-like molecules lacking the hydrophilic arms, **1** and **2** (IV, Fig. 1) were employed. By using differential scanning fluorimetry (DSF) we showed that 10 μM **1** was able to bind to the 5 μM TC37, based on the change of the fluorescence profile between samples with and without the ligand (IV, Fig. 5D). Addition of 10 μM **2** also changed the fluorescence profile but not as markedly as in the case of **1** (IV, Fig. 5E). The effect of double mutations (E8.11A and E24.28A) on TC37 was studied next. The addition of 50 μM mitoxantrone to the 5 μM double mutants had little effect on the fluorescence profile, indicating that the mutations impair the affinity of mitoxantrone to TCPTP (IV, Fig. 5B-C). Instead, addition of 10 μM **1** and **2** impacted more on the fluorescence profiles of mutated TCPTP's (IV, Fig. 5D-E). This suggests that **1** and **2** are able to bind mutated TCPTP and that E8, E11, E24, and E28 are not as important for the binding of **1** and **2** as they are for the binding of mitoxantrone. The DSF measurements also suggested that E8.11A mutant is less stable than E24.28A mutant and TC37.

6 DISCUSSION

This thesis comprises studies of proteins from separate protein families that are all important for human health. Whereas VAP-1's function can be inhibited using small molecular inhibitors, TCPTP's function can be activated using small molecules. Cystic fibrosis is caused by mutations that disturb the natural protein-protein interactions. The methods used in the research described in this thesis include most steps needed in rational drug discovery (Fig. 1). The development of computing resources has revolutionized the structural study of proteins, as they have become more available. When used correctly, computational approaches can save considerable time and money.

The results acquired by using computational methods need to be reviewed and validated carefully and objectively, as it is possible for a skilled modeler to influence the successful outcome of the calculations. More importantly, revision is needed because, even though algorithms and parameters used in computational methods are good they still are unable to describe biological and chemical events perfectly. For example, the hydrazine molecules, studied in articles I and II of this thesis, bind covalently to TPQ and the direction of the covalent bond is parallel to the plane of the aromatic ring of the TPQ. Understandably, the docking softwares are unable to create the constriction of bond rotation caused by resonance. Thus, the docking results did not entirely represent the biological situation. The scoring functions implemented in the docking softwares can also emphasize some atomic interactions over the others, and thus, the best conformation according to the scoring function is not necessarily the best in reality. Nevertheless, the docking results provided reasonably good starting conformations in articles I, II and IV.

The 3D QSAR models built in article I went through multiple validation steps: 1) the division of molecules into subsets that were predicted by using submodels, 2) bootstrapping, 3) progressive scrambling, and 4) models were used to predict the biological activities of five newly synthesized molecules. All five molecules were predicted reasonably well, even those that were structurally different to the molecules used in the model building. This was rather surprising but it supported the validity of built models.

The targets for docking simulations in this thesis were mostly experimentally determined crystal structures retrieved from the PDB. In article II, some docking simulations were performed on a structure obtained from the MD. Even though 3D structures are carefully validated prior to their submission to and release in the PDB, they should not be assumed to be the "only truth". A crystal structure only represents the conformation in which the protein has crystallized and it may not represent the biologically occurring conformation. This is especially relevant in the case of large proteins comprising multiple domains crystallized individually, such as filamin. It also should be acknowledged that proteins are dynamic and they are able to modify their binding sites according to the ligands they bind, a process called induced fit. Therefore, the docking of molecules inside a structure in which the co-crystallized ligand has been removed might not describe the binding between protein and docked ligand accurately, since the conformation of the protein is kept static during docking simulations.

MD is a method that can be used to predict the conformational changes that occur as new molecules are introduced *via* docking simulations to either *apo* structures or to structures from which the bound ligands have been removed. Ligands need to be minimized and parameterized prior to the actual MD simulation. The parameters are empirical data and thus they describe actual events, but they are also a source of error. The atoms move time-dependently in MD simulation according to the force field that has been selected by the user. The selection of a force field should be considered carefully because different force fields have been developed for different needs.

MD simulations can introduce conformational changes to the protein structure that cannot be deduced merely by visualization of the static 3D structure on the computer screen. MD has proven to be a very useful tool in articles I and II, as the ligand-binding pocket of VAP-1 was significantly expanded in response to ligand binding compared to the ligand-binding pocket seen in the 3D crystal structure of VAP-1 retrieved from the PDB (Jakobsson et. al. 2005, Airenne et. al 2005). Furthermore, the conformational changes seen in VAP-1 simulations are unlikely to occur in DAO, based on the visualization of crystal structures of VAP-1 and DAO and the comparison of their corresponding amino acids. Thus, valuable information about the subtype selectivity of VAP-1 was obtained.

The lack of an experimental 3D structure does not mean that the protein of interest cannot be studied. If a structure of a closely related protein is available, then homology modeling can be used. The greater the sequence identity between the sequence of the studied protein and the sequence of the template, the more accurate the model should be. Homology modeling was used in article IV to build as many missing amino acids onto both the N- and C-termini as possible. Docking of mitoxantrone, followed by MD simulations, was initially done to the crystal structure, but the N-terminus of the crystal structure (Ile5) interfered with the ligand and, therefore, the missing amino acids needed to be added to the model.

Proteins can be studied based on their sequences or by combining sequence information with structural information. In article III the sequences of the FLNa domains were aligned and the sequence differences in CD-faces between different domains were investigated. Structural information was also introduced to the alignment which substantially increased the reliability of the alignment. The validity of the sequence alignment is sometimes difficult to establish, as the alignment programs are able to align any given sequences even though they are not homologous proteins. The results described in article III give information about the subdomain selectivity of different filamin domains. In tandem with article III an experimentally determined structure of FLNa domain 21 in complex with S13F was published (Smith et al. 2010). The structure contained interactions which were predicted in article III, which confirmed the modeling results.

In article IV the binding site of mitoxantrone in TCPTP was identified by using computational methods in combination with experimental methods. TCPTP is an important antitumor factor, as it negatively regulates many signaling proteins involved in malignancies. The recognition of an activator binding site is therefore an important starting point for the development of novel antitumor agents.

The binding of mitoxantrone and spermidine to TCPTP was studied experimentally using ITC and DSF. It was clearly demonstrated that spermidine did not bind to the catalytic domain of TCPTP but mitoxantrone did. The putative binding site was determined by using molecular modeling. Based on the knowledge that all reported binding partners of TCPTP possess a positive charge, an area which contained two pairs of negatively charged glutamate residues (E8, E11, E24, and E28) at the opposite sides of a hydrophobic groove was identified. The putative binding site was verified by mutating E8, E11, E24, and E28 to alanine residues. The mutation of glutamate residues to alanine residues could not inhibit the binding of mitoxantrone completely, according to the DSF experiments, but the mutations destabilized TCPTP.

In DSF the change in the protein melting is recorded as the amount of fluorescence as a function of temperature. Neither mitoxantrone, the buffer, nor any of the proteins possessed autofluorescence in the wave length used in the DSF studies; therefore the sources of background were minimal. It is, however, possible that the strongly-colored mitoxantrone is able to absorb the fluorescence of the fluorophore and thus reduce the detected fluorescence signal.

The results in this thesis were acquired by using a combination of computational and experimental methods. Diabetes and cancer are diseases that are amongst the main causes of death in western civilization and enormous amounts of time and money are spent in order to find cure for these diseases. The results in this thesis are important for the future development of pharmaceuticals for diabetes, cancer, and cystic fibrosis.

7 CONCLUSIONS

The main conclusions of this thesis, acquired by combining computational and experimental methods, are:

1. The factors which contribute to the selective VAP-1 binding of hydrazines were identified. The dynamic nature of the VAP-1 ligand-binding pocket and the novel extra space inside VAP-1 were also discovered. These results give novel insight to the VAP-1 selectivity over other AOs, which should be taken into consideration when new VAP-1 inhibitors are being designed.

2. The basis of subdomain selectivity of the FLNa domains was studied. The sequence length and amino acid composition between the strands C and D contribute to the selectivity. The mis-sense mutation S13F containing CFTR is unable to bind to the CD-face because: 1) serine participates in important hydrogen bonding, and 2) phenylalanine introduces steric hindrance.

3. The binding site for mitoxantrone in a potent anti-cancer target TCPTP was identified to be a hydrophobic groove near the N-terminus. The main component of the binding is entropy and there is one binding site for mitoxantrone per one TCPTP molecule. The glutamate residues at the opposite sides of hydrophobic groove contribute to the stability of TCPTP and their mutation into alanine residues has a negative effect on the binding of mitoxantrone.

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YHTEENVETO (RÉSUMÉ IN FINNISH)

Rationaalinen lääkeainesuunnittelu – Proteiini-ligandi rakennekokonaisuuksien tutkimus

Rationaalinen lääkeainesuunnittelu on yhdistelmä kokeellisia ja laskennallisia menetelmiä, joissa hyödynnetään proteiinien kolmiulotteisia rakenteita. Tässä työssä rationaalisen lääkeainesuunnittelun kohteena on ollut kolme eri proteiinia. Työssä on pyritty kehittämään lääkeainemolekyylejä syöpää ja diabetesta vastaan. Myös kystisen fibroosin aiheuttavaa mutatoitunutta proteiinia on tutkittu rakennetasolla.

Vaskulaarinen adheesioproteiini 1 (VAP-1) on verisuonten endoteelisolujen pinnalla esiintyvä proteiini, jonka roolina on auttaa valkosolujen pääsyä verisuonista tulehtuneisiin kudoksiin. VAP-1 toimii myös entsyyminä, joka muuntaa elimistön luonnollisia, sekä vieraita amiiniyhdisteitä aldehydeiksi. Reaktiossa vapautuu myös sivutuotteita, jotka korkeissa konsentraatioissa ovat elimistölle myrkyllisiä, ja voivat aiheuttaa esim. diabetes- ja tulehdusoireita.

Tässä työssä tutkittiin hydratsiiniyhdisteiden sitoutumista VAP-1:een sekä muihin samansukuisiin proteiineihin, mm. monoamiinioksidaaseihin (MAO) sekä diamiinioksidaasiin. Tarkoituksena oli kehittää ainoastaan VAP-1:een sitoutuvia hydratsiinimolekyylejä. Ensimmäisessä osajulkaisussa rakennettiin kolmiulotteiset kvantitatiiviset rakenne-aktiivisuus-mallit käyttäen 47 hydratsiinimolekyyliä, sekä niiden IC_{50} arvoja sekä VAP-1:lle että MAO:lle. Mallien avulla pystyttiin päättelemään mitkä ominaisuudet hydratsiiniyhdisteissä ovat hyödyllisiä sekä VAP-1 että MAO sitoutumisen kannalta. Mallien pohjalta pääteltiin, että vety on paras substituentti positiossa R1 (I, Taulukko 1) ja että metyyliä isommat substituentit samassa positiossa aiheuttavat VAP-1 selektiivisyyden menetyksen. Työssä huomattiin myös, että VAP-1:n ligandinsitomistaskussa tapahtuu mielenkiintoinen konformaation muutos, jonka johdosta ligandinsitomistaskuun muodostuu lisätilaa.

Osajulkaisussa II edellisen työn malleja käytettiin hyödyksi 9 uuden VAP-1 estäjän suunnittelussa. Uusia hydratsiinimolekyylejä käytettiin tutkimaan VAP-1:n ligandinsitomistaskun dynamiikkaa. Työssä havaittiin sama taskun kasvaminen, kuin I osajulkaisussa. Lisäksi havaittiin, että R1 position metylointi yhdessä ison R2 substituentin kanssa lisäsi sitoutumisaffiniteettiä VAP-1:een muttei MAO:on (II, Taulukko 1), jonka johdosta selektiivisyys VAP-1:een kasvoi verrattuna niihin molekyyleihin, joilla oli vety R1 positiossa.

Kolmannessa osajulkaisussa tutkittiin mutatoituneen CFTR (*engl.* cystic fibrosis transmembrane conductance regulator) proteiinin ja filamiini A:n välisen sitoutumisen rakenteellista perustaa. Havaittiin, että CFTR sitoutuu vain sellaisiin filamiini A:n domeeneihin, joilla on tietynkaltainen ja -pituinen aminohappojärjestys C- ja D-juosteiden välissä. S13F CFTR ei pysty sitoutumaan filamiini A:han, mikä johtuu kahdesta syystä: 1) S13 muodostama vetysidos

häviää, ja 2) F on isompi, kuin S, mistä johtuen mutatoitunut CFTR ei mahdu sitoutumaan samalla tavoin C- ja D-juosteiden väliin, kuin normaali CFTR.

Osajulkaisussa IV tutkittiin aiemmin tunnistetun aktivaattorimolekyylin, mitoxantronen, sitoutumista TCPTP-proteiiniin (*engl.* T-cell protein tyrosine phosphatase). TCPTP on potentiaalinen syöpälääkekehityskohde. Kokeellisesti pystyttiin osoittamaan, että mitoxantrone sitoutuu TCPTP:hen. Mallinnuksen avulla tunnistettiin potentiaalinen sitomispaikka TCPTP:n pinnalla. Sitoutumispaikka pystyttiin varmistamaan tutkimalla kokeellisesti sekä laskennallisesti proteiineja, joissa oli mutatoitu muutamia sitoutumispaikalla sijaitsevia oletetusti tärkeitä aminohappoja. Työssä löydettiin myös kaksi uutta potentiaalista TCPTP-aktivaattoria, joiden pohjalta jatkossa pystytään suunnittelemaan paremmin sitoutuvia aktivaattoreita.

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