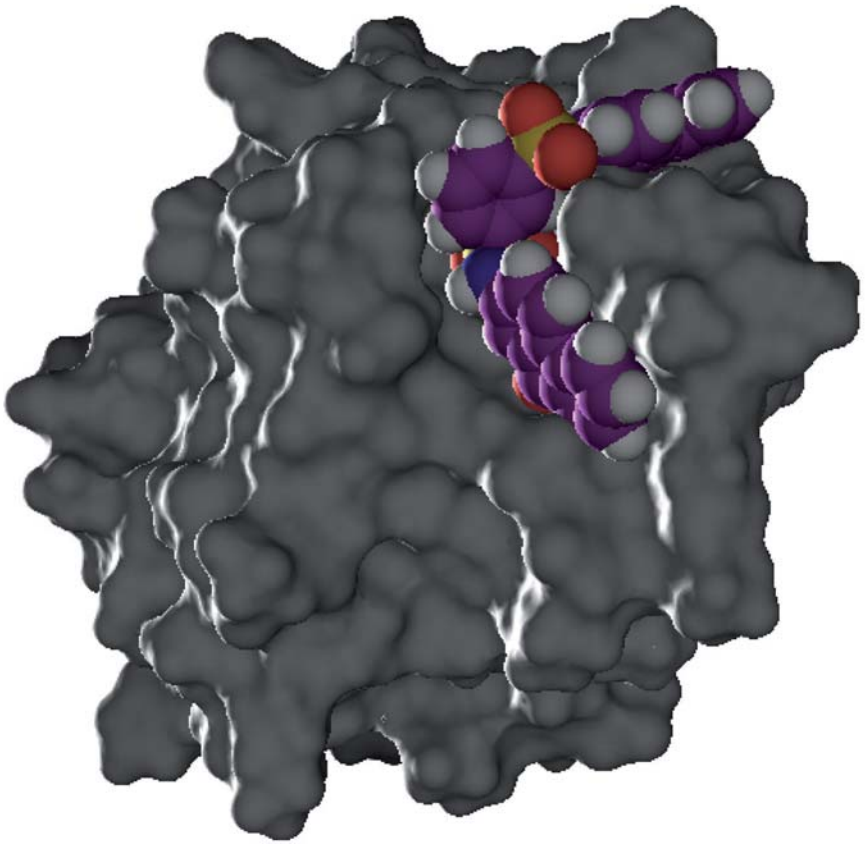
JYVÄSKYLÄ STUDIES IN BIOLOGICAL AND ENVIRONMENTAL SCIENCE
224

Jarkko Koivunen

Discovery of α2β1 integrin ligands: Tools and drug candidates for cancer and thrombus



JYVÄSKYLÄN YLIOPISTO

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UNIVERSITY OF JYVÄSKYLÄ

JYVÄSKYLÄ 2011

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"But it ain't about how hard ya hit. It's about how hard you can get it and keep moving forward. How much you can take and keep moving forward."

-Rocky Balboa

ABSTRACT

Koivunen, Jarkko
Discovery of α2β1 integrin ligands: Tools and drug candidates for cancer and thrombus.
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Yhteenveto: α2β1-integriiniligandien suunnittelu; lääkeaihioita ja työkaluja syövän ja veritulpan hoitoon
Diss.

Integrins are cell-surface proteins that mediate signalling between the cytoplasm and the extracellular matrix, and furthermore, attach cells to their surroundings. Four of these proteins, α1β1 α2β1 α10β1, and α11β1, act as collagen receptor. α2β1 integrin–collagen interaction has shown to have a role in several pathological processes such as thrombus and cancer. Accordingly, recent studies have indicated that the α2β1 integrin is a promising target for drug discovery. However, only a few potential drug candidates have been found so far. The understanding of the α2β1 integrin structure-function relationships, which are yet partially unknown, is crucial when novel pharmaceuticals are developed. In this thesis, several novel α2β1 integrin function modulating ligands were developed by using computer-aided target-based drug discovery. First, a set of molecules was rationally designed, synthesized, and their biological activity was tested to shed light to structure-activity relationships of effective α2β1 integrin–collagen I inhibitors. Achieved results were used in the development of a fluorescent tool molecule. This fluorescent probe was used to explore the α2β1 integrin function in cellular level, and also in identifying the binding mode of novel α2β1 ligands. The small molecule crystal structures provide information about intermolecular interactions that ligands can potentially form. Thus, several crystal structures of discovered integrin ligands were successfully solved. In the future, these structures can be utilized when novel α2β1 ligands are sought by using virtual screening. This thesis gives valuable insight to the α2β1 integrin ligands and is an important part of a journey to safe and effective integrin pharmaceuticals.

Keywords: α2β1 integrin; collagen receptor integrins; rational drug discovery; thrombus; X-ray crystallography.

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CONTENTS

LIST OF ORIGINAL PUBLICATIONS

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

I Koivunen J.T., Nissinen L., Juhakoski A., Pihlavisto M., Marjamäki A., Huuskonen J. & Pentikäinen O.T. 2011. Blockage of Collagen Binding to Integrin α2β1: Structure-activity Relationship of Protein-protein Interaction Inhibitors, *MedChemComm* doi: 10.1039/c1md00089f (in press).

II Koivunen J.T., Nissinen L., Käpylä J., Pihlavisto M., Heino J., Marjamäki A., Huuskonen J. & Pentikäinen O.T. 2011. Fluorescent probe to modulate and explore α2β1 integrin function. Submitted manuscript.

III Korhonen J., Marjamäki A., Nissinen L., Pihlavisto M., Pentikäinen O.T. & Koivunen J.T. 2010. Urea substituted sulphonamide derivatives. *Patent application:* WO/2010/146236.

IV Nissinen L., Koivunen J.T., Käpylä J., Nieminen J., Jokinen J., Sipilä K., Pihlavisto M., Pentikäinen O.T., Marjamäki A. & Heino J. 2011. Under shear stress α2β1 integrin binding to collagen does not require receptor pre-activation. Submitted Manuscript.

RESPONSIBILITIES OF JARKKO KOIVUNEN IN THE THESIS ARTICLES

Article I I designed, synthesized and characterized the molecules. I wrote the article with Olli Pentikäinen. I prepared all of the figures.

Article II I designed, synthesized and characterized the molecule. I solved the crystal structures together with Juhani Huuskonen. I planned and performed the spectroscopy studies and took part in confocal microscopy studies. I participated in planning the FACS studies. I wrote the article together with Olli Pentikäinen and prepared the figures together with Olli Pentikäinen and Liisa Nissinen.

Article III I participated in developing the molecules together with other authors.

Article IV I took part in developing the molecules together with other authors. I planned and performed the DSF studies and prepared the figures from the DSF results. I participated in planning and writing the article.

All the studies in this thesis were performed under the supervision of Olli Pentikäinen and Juhani Huuskonen.

ABBREVIATIONS

DAP	2,3-diaminopropionic acid
DSF	Differential scanning fluorimetry
EC_{50}	Half maximal effective concentration
ECM	Extra cellular matrix
FACS	Fluorescence activated cell sorting
IC_{50}	Half maximal inhibitory effect
ICAM-1	Inter Cellular Adhesion Molecule 1
MIDAS	Metal ion depended adhesion site
MMP-1	Matrix metalloproteinase-1
MT1-MMP	Membrane Type 1 metalloproteinase
Pro	Prolyl-sulfonamide
RGD	Arginine-Glysine-Aspartatic acid
RKKH	Arginine-Lysine-Lysine-Histidine
SEM	Standard error of measurement

1 INTRODUCTION

Integrins are widely explored proteins located at the cell-surface. They attach cells to their environment and mediate signalling between the cytoplasm and the extracellular matrix. To date, the complete understanding of structure- function relationships of integrins is largely unsolved. Four of 24 human integrins act as collagen receptors. Collagen is the most abundant protein in human, thus, this interaction participates in many biological events, and furthermore, in some pathological processes such as thrombosis and cancer metastasis. The α2β1 integrin belongs to subgroup of collagen receptor integrins and has proved to be promising target when treatment for thrombus and cancer are sought.

Recent studies indicate that it is possible to rationally develop small molecule ligands to block the collagen binding into blood platelets via the α2β1 integrin. The blockage of collagen-α2β1 integrin interaction can be used as a novel approach for antithrombotic therapy. In addition to this, several studies have shown that collagen-α2β1 integrin interaction has a role in cancer metastasis. In order to develop safe and effective drugs which target this interaction, structure-activity relationships of potential drug candidates should be explored. Additionally, the α2β1 integrin function and signalling process should be understood at the atomic level before effective pharmaceuticals can be developed.

This thesis focuses on the discovery of novel α2β1 integrin ligands that disturb the collagen binding. Computer-aided molecular discovery was used as the primary tool when ligands were developed. As result, a set of molecules was designed in order to understand the structure-activity relationships of α2β1–collagen I inhibitors. Furthermore, some of the discovered molecules were found to be valuable tools for solving the puzzle of integrin signalling. To study ligands further, several small molecule crystal structures were solved. Crystal structures provide information about intermolecular interactions that molecules can form. Moreover, the experimental data collected from structures can be used to validate computer-aided theoretical methods. Additionally, small molecule crystal structures can be used when virtual screening methods for ligand discovery are developed.

2 REVIEW OF THE LITERATURE

2.1 Integrins

Integrins are heterodimeric cell-surface proteins that participate in many biological and pathological functions, such as wound healing, angiogenesis, inflammation, metastasis and thrombosis (Hynes 1987, Hynes 2002, Gahmberg et al. 2009). There are totally 24 known different human integrins formed by 18α and 8β units (Fig. 1). The integrin heterodimer formed by one α- and one β-subunit is held together with noncovalent interactions. The general structure of integrins contains a large extracellular part, transmembrane domains, and relatively short cytoplasmic tails (Fig. 2) (Hynes 2002).

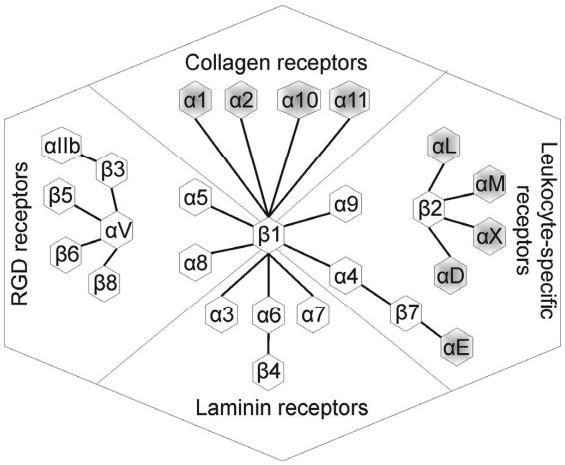


FIGURE 1 The 24 heterodimers of the integrin receptor family. Nine αI containing integrins are shown in grey. Figure is based on Barczyk et al. (2010).

Upon signalling and activation events integrins undergo conformational changes from "bent" to "extended" conformation according to the activity stage (Fig. 1) (Xiong et al. 2001, Takagi et al. 2002, Shattil et al. 2010, Nishida et al. 2006). The activated, extended conformations can have a closed, intermediate or open headpiece. The extending and the high affinity conformation is presumed to require separation of the transmembrane parts, and as a result, the cytoplasmic tails (Lu et al. 2001, Vinogradova et al. 2002, Beglova et al. 2002, Takagi et al. 2002). Conventionally, the general assumption is that the integrins in the "bent" conformation are incapable to bind macromolecular ligands. However, it is shown that the bent integrins can bind large ligands (Jokinen et al. 2010), and some peptides (Xiong et al. 2002, Adair et al. 2005). A snake venom protein, rhodocetin, is also assumed to bind to the low-affinity conformation of the integrin (Eble & Tuckwell 2003). Furthermore, it ihas been presented that the integrins can recognize ligands without prior activation (Siljander et al. 2004). Nevertheless, it is still unproven whether the ectodomain needs to extend for the high affinity state of the integrin or not (Gahmberg et al. 2009).

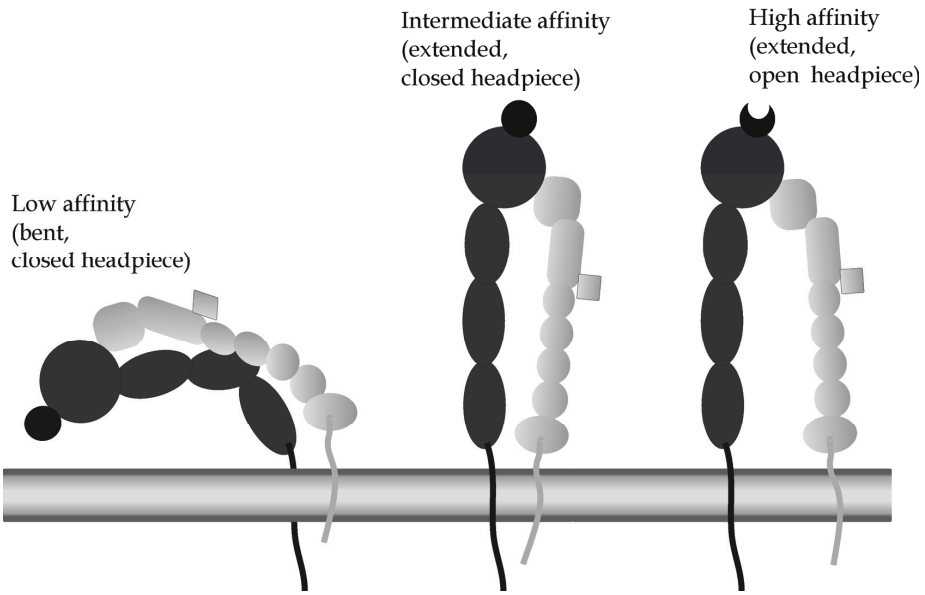


FIGURE 2 Schematic view of the integrin in different conformations. The importance of extending the complete integrin to achieve high affinity is under debate. The figure is based on Gahmberg et al. (2009).

2.1.1 Integrin therapeutics

Despite the essential importance of the integrins in human health, up to date only five integrin inhibitors have received marketing approval (Cox et al. 2010).

Even tough they have been approved for marketing, all pharmaceuticals targeting integrins have major restraints.

Three intravenous antiplatelets targeting platelet integrin αIIbβ3; an antibody abciximab (EPILOG investigators 1997, Umans et al. 1997), a cyclic peptide eptifibatide (Scarborough 1999) and a small molecule tirofiban (The RESTORE Investigators 1997, PRISM-PLUS Study Investigators 1998), were the first pharmaceuticals in the market that act as integrin function modulators (Fig. 3). However, the efforts for developing drugs further from intravenous to oral intake have failed. An additional problem was the agonists-like activity expressed by the integrin ligands. This caused problems in potency and in pharmacology of these drugs. Thus, the first drugs targeting integrins were not success stories despite the promising start.

Two integrin therapeutics have been developed to treat autoimmune diseases. A monoclonal antibody targeting the α4 integrin, named natalizumab, was approved for the treatment of Crohn´s disease (Targan et al. 2007) and multiple sclerosis (Rice et al. 2005). Another monoclonal antibody, efalizumab, which binds to the αLβ2 integrin, was accepted as a treatment for psoriasis (Frampton & Plosker 2009). Both of these had severe side effects that led to market withdrawal. However, natalizumab was brought back to the market with a risk-management strategy (Cox et al. 2010).

Incomplete understanding of the integrin signaling caused unwanted side effects and led to problems with the integrin targeted pharmaceuticals. Many of the candidates were designed based on the natural ligands of the integrins, thus, many of them work as partial agonists i.e. the ligands inhibited binding to the RGD motif, but activated the integrin signaling cascade (Weis et al. 2009). For example, some αIIbβ3 antagonists based on the RGD motif, inhibited the platelet aggregation at high doses, while low doses induced it (Quinn et al. 2000, Cox et al. 2000). Although integrins have been shown to be extremely potent targets for drug development, their complexity and importance in biological mechanisms is not understood properly. Therefore, more knowledge regarding the integrin structure and function is needed before effective and safe integrin pharmaceuticals are discovered.

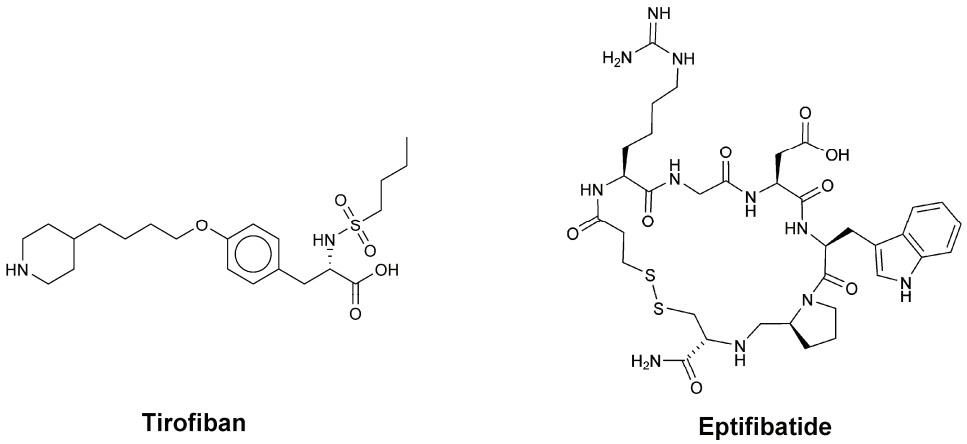


FIGURE 3 The αIIbβ3 integrin drugs approved for marketing.

2.2 Collagen receptor integrins

Nine of the 24 human integrins include an inserted, independently folding ~200 amino acids long αI domain which mediates the ligand binding (Fig. 1) (Bahou et al. 1994, Tuckwell et al. 1995, Calderwood et al. 1997, Velling et al. 1999). This domain is also called an A domain due to the likeness with the von Willebrand factor A domain (Lee et al. 1995). Interestingly, the αI domain is found only in Chordates, which indicates that the I domain was developed relatively late in evolution (Hughes 2001, Huhtala et al. 2005). The subgroup of the αI domain containing integrins is divided into two categories; leucosyte specific and collagen receptor integrins (Hynes 2002). In total, these are four integrins that recognize collagens: α1β1, α2β1, α10β1 and α11β1 (Takada et al. 1988, Briesewitz et al. 1993, Tuckwell et al. 1995, Camper et al. 1998, Velling et al. 1999). On the contrary to the collagen receptor integrins, the other β1-associated integrins do not contain the I domain (Fig.1).

As their name indicates, the collagen receptor integrins bind various collagens (Kern et al. 1994, Tuckwell et al. 1995, Dickeson et al. 1999, Tiger et al. 2001 Tulla et al. 2008). Different integrins favour different types of collagens; for example the α2β1 and the α11β1 have high affinity for fibril-forming collagens such as collagen I (Tulla et al. 2001, Tulla et al. 2008). In contrast, the α1β1 and the α10β1 favour collagen IV (Kern et al. 1993, Tulla et al. 2001, Tulla et al. 2008). In addition to this, several different proteins act as physiological, extracellular ligands of the collagen receptor integrins. For example, they act as receptors for different laminins (Colognato-Pyke et al. 1995, Colognato et al. 1997, Tulla et al. 2001, Tulla et al. 2008).

The natural ligands of the α2β1 have been widely explored. Likewise to the α10β1 and the α1β1 integrins, also the α2β1 binds laminins (Elices & Hemler 1989, Languino et al. 1989, Colognato et al. 1997, Tulla et al. 2008). Furthermore,

the α2β1 binds to chondroadherin (Camper et al. 1997), decorin (Guidetti et al. 2002), and tenascin C (Sriramarao et al. 1993). Some pathogens, such as echo virus 1 and some rotaviruses, use the α2β1 integrin as a receptor (Bergelson et al. 1992, Coulson et al. 1997, Hewish et al. 2000, Zárate et al. 2000). The selectivity over different ligands depends on the activation state of the corresponding integrin (Käpylä et al. 2000, Tulla et al. 2001, Tulla et al. 2008). On the contrary to some integrins, the collagen binding integrins do not recognize the RGD binding motif (Fig. 1). Instead, the collagen receptor integrins bind to the GFOGER sequence (O refers to hydroxyproline) of the collagen mimic peptide (Knight et al. 2000, Emsley et al. 2000). The triple helical conformation of the collagen peptide, as well as the glutamate residue, were found to be essential for the recognition (Morton et al. 1994, Knight et al. 2000). It was found that replacing glutamate with the comparatively similar aspartate causes a total loss of binding. Also, 4-hydroxyproline instead of proline is essential for binding of the α1β1 but not for the α2β1 (Perret et al. 2003). Interestingly, in some cases the isolated recombinant I domain showed different binding properties when compared to the cellular adhesion tests (Knight et al. 2000). The K_d (7.8μM) has been determined for the mimetic peptide of collagen (Lambert et al. 2008). Furthermore, some other sequences are assumed to be recognized by the α2β1 integrin (Knight et al. 2000).

In addition to the ECM ligands, integrins recognize various cytosolic proteins. Talin and kindlin are ligands of cytosolic parts of the β subunit which trigger the inside-out activation of integrins (Hynes 2002, Ye et al. 2010). Other intracellular integrin ligands are protein kinase C (Ng et al. 1999) and 14-3-3, that acts as an integrin activator (Takala et al. 2008). Filamin is shown to act as a negative intracellular regulator of integrins (Calderwood et al. 2001, Lad et al. 2007). Furthermore, phosphorylation of the cytoplasmic tails has a major role in integrin regulation (Gahmberg et al. 2009).

The ectodomain of the integrins contain several different binding sites for divalent cations that are presumed to regulate the affinity and function of the integrins (Xiong et al. 2001). The β1 domain contains three cation binding sites: the metal ion-dependent adhesion site (MIDAS), adjacent to MIDAS (ADMIDAS), and ligand-associated metal-binding site (LIMBS) (Xiong et al. 2001, Valdramidou et al. 2008). Additionally, the αI domain contains a MIDAS as the main binding site for physiological ligands. Magnesium and manganese ions seem to adjust integrin to the extended, high-affinity conformation whereas high concentrations of calcium ions have been represented to act as negative regulators (Xiong et al. 2001, Takagi et al. 2002, Chen et al. 2003). However, at low concentrations Ca^{2+} together with Mg^{2+} ions can stimulate integrin depended adhesion (Mould et al. 2003).

The crystal structures of the I domains of leukocyte-specific integrins αM (Lee et al. 1995, Lee et al. 1995, Baldwin et al. 1998), αL (Qu & Leahy 1995) and αX (Vorup-Jensen et al. 2003) have been solved. Additionally the αLI domain has been crystallized in complex with lovastatin (Kallen et al. 1999) and with ICAM-1 (Shimaoka et al. 2003). Furthermore, the I domain crystal structures of

the collagen receptor integrins α1 (Nolte et al. 1999, Rich et al. 1999, Salminen et al. 1999, Nymalm et al. 2004) and α2 (Emsley et al. 1997, Emsley et al. 2000) have been reported. Recently, the crystal structure of a complete αXβ2 ectodomain with the αI domain was published (Xie et al. 2010).

2.2.1. The α2β1 integrin

The α2β1 is the most abundant integrin on the surface of platelets. Furthermore, the α2β1 is also expressed in fibroblasts, epithelial and endothelial cells (Zutter and Santoro 1990). The α2β1 integrin-collagen interaction is presumed to be a crucial element in development of thrombus (Sweeney et al. 2003), and in spreading of cancer cells (Guo & Giancotti 2004). For example, a prostate cancer metastasis is proven to be mediated by the α2β1 integrin-type I collagen interaction (Hall et al. 2006). Furthermore, it has been speculated that the α2β1 antagonists may have potential in therapeutic applications in the prevention of bone metastasis associated with prostate cancer (Goel et al. 2008). Studies have also proposed that the α2β1 integrin may be a target for the treatment of pancreatic cancer (Grzesiak & Bouvet 2006), as well as rhabdomyosarcoma (Chan et al. 1991). Novel molecular tools to explore the α2β1 and the blockage of collagen-α2β1 interaction could explain the significance of this integrin as a drug target.

2.2.2. The α2I domain

The I domains bind to their ligands mainly via the MIDAS (Fig. 4) (Michishita et al. 1993). Two crystal structures of the α2I domain, one with a collagen peptide (Emsley et al. 2000) and one without it (Emsley et al. 1997), reveal that the I domain undergoes a large conformational change when it forms a complex with the collagen (Fig. 4A). The conformational transformation from the "closed" unbound conformation to the "open" collagen binding conformation significantly increases the collagen binding affinity of the αI domain (Lu et al. 2001, Shimaoka et al. 2001). Consequently, the "open" and "closed" conformations are usually described as the high-affinity and the low-affinity conformations, or active and inactive, respectively. Both conformations exist on the cell surface although the closed conformation is supposed to be preferred (Li et al. 1998, Shimaoka et al. 2001, Shimaoka et al. 2002, Van de Walle et al. 2005, Cruz et al. 2005).

The major activation-induced conformational change in the I domain is the uncoiling of the αC helix at the same time as the α6 helix gains an extra turn (Emsley et al. 2000). As a result of the αC helix unwinding, the position of residues 284–288, located in the MIDAS region, change radically. Simultaneously, the C-terminal helix α7 moves towards the βI domain and this shift breaks the salt bridge between E318 and R288. The downward movement makes E336 of the α7 helix able to interact with the βI domain. Furthermore, it is suggested that E336, a highly conserved residue, could act as ligand of the βI

and coordinate to Mg^{2+} in MIDAS of the βI. This way, the activated βI domain may draw the α7 helix downwards and activate the αI domain. Thus, the ligand induced outside-in signalling is presumed to be controlled via the activated βI domain (Shimaoka et al. 2002). Conversely, the activation state and conformation of the αI domain is represented to be allosterically regulated by the conformational changes in the β subunit caused by the inside-out signalling (Shimaoka et al. 2002, Yang et al. 2004). However, recent studies with the αXβ2 I domain-containing integrin indicated that the contact between the αI and the βI domains is more flexible than formerly supposed (Xie et al. 2010). The linker between the αI domain and the β subunit is highly conserved, therefore, it is presumable that also the α2I domain contains similar flexibility. The conformational changes of the αI domain eventually trigger the whole outside-in signalling cascade and lead to the separation of transmembrane and cytosolic domains. This enables the binding of cytosolic ligands to integrins, and finally leads to the inside-out signalling initiated by the cytosolic ligands (Takagi et al. 2002).

In order to explore binding affinity and function of the different conformations of the integrins several mutations of essential amino acids have been developed. Mutation E310A in αL or E320A in αM is presumed to drive the integrin to the bent conformation with a "closed" headpiece (Fig. 2; Alonso et al. 2002, Shimaoka & Springer 2003, Yang et al. 2004, Salas et al. 2004). The corresponding mutation in the α2I domain is E336A (Connors et al. 2007, Jokinen et al. 2010). Mutation of E318W or E318A in the α2β1 integrin is supposed to present the activated, extended integrin with an "open" headpiece (Fig. 2; Aquilina et al. 2002). Additionally, the presence of calcium ions has been represented to favour the in-active conformation, whereas the presence of manganese or magnesium is supposed to favour the activated state of the integrins (Xiong et al. 2001, Takagi et al. 2002, Eble & Tuckwell 2003). Furthermore, some antibodies have shown to recognize only the active conformation of the α2β1 (Oxvig et al. 1999).

Recent studies are changing the traditional conclusion according to which the "standing", extended, integrins are able to bind ligands while the "crouched" ones stay inactive. Furthermore, the contact of the I domain with the other parts of integrin is found to be highly flexible. However, the starting point of the collagen binding has not been solved thoroughly. The complete conclusion about the integrin function under flow and static conditions, the total influence of the inside-out and outside-in signaling to the collagen recognition, as well as effect of the bent and extended conformations on the conformation of the I domain still remain unverified.

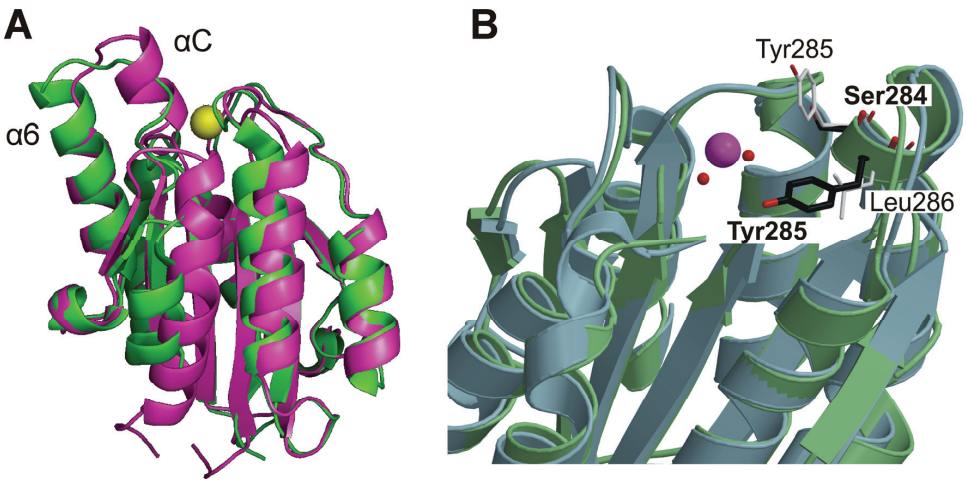


FIGURE 4 (A) Superimposed crystal structures of the α2I domain in the "open" (green, PDB code: 1DZI) and the "closed" (cyan, PDB code: 1AOX) conformation. (B) Superimposed I domains of the α2 (green, PDB code: 1AOX) and the α1 (blue, PDB code: 1PT6) showing the main differences of the binding site: Tyr285 of α2 (grey) is Ser284 (black) in α1, Leu286 of α2 (grey) is Tyr285 (black) in α1, respectively.

2.3 Collagen I-α2β1 integrin inhibitors

Attempts to understand the meaning of collagen binding in the development of pathological events has so far yielded various types of ligands to interfere the α2β1 integrin-collagen interaction. Few natural proteins have been found to inhibit the α2β1 binding to collagen. The leech product saratin inhibits the collagen-platelet interaction under shear via von Willebrand factor (Barnes et al. 2001) and also via the α2β1 integrin (White et al. 2007). Another natural product, rhodocetin, acts as an antagonists of the α2β1 integrin (Eble et al. 2002). The binding site of rhodocetin was proposed to be in the α2I domain and analogous with collagen (Eble & Tuckwell 2003). Moreover, rhodocetin was found to favour acidic pH and the presence of calcium ions, that is, circumstances where integrin is assumed to be inactive.

A small molecule inhibitor called AJP117510 (Fig. 5), a nucleoside derivative isolated from fungus, was reported to inhibit collagen binding to the α2I domain with an IC_{50} value of 5.9 μM (Sato et al. 2006). The article contained neither speculations about the binding site or the binding mode nor results about selectivity.

2.3.1 Peptides

A cyclic CTRKKHDC peptide derived from the Jararhagin, snake venom of Brazilian viper Bothrops jararaca was found to inhibit the collagen–α2β1 integrin interaction (Ivaska et al. 1999, Pentikäinen et al. 1999). This peptide was shown to be dependent on five negatively charged amino acid residues of the α2I domain. However, the deletion of the C helix of the αI domain did not affect on the binding of the RKKH peptide. Interestingly, the binding of the RKKH peptide was Mg^{2+} dependent, although the deletion of the C helix and this way a major alteration of the MIDAS do not have an influence on the binding of the peptide. Furthermore, molecular modeling proposed that the peptide does not directly interact with Mg^{2+} (Pentikäinen et al. 1999).

Contradictory results have been represented about the influence of the peptide to the conformation of the I domain. In the α1 integrin, it was suggested that the RKKH peptide would change the conformation of the α1I domain (Nymalm et al. 2004). However, the NMR studies with the homologous α2I domain did not support these results (Lambert et al. 2008). So far, there is no evidence that the RKKH peptide would produce a collagen-like signaling event in platelets. However, the jararhagin binding to the α2β1 in fibroblasts triggers collagen-like cell signaling events such as up-regulation of MMP-1 and MT1-MMP (Zigrino et al. 2002). The distinct behavior of the same protein-ligand complex in different cell types proves how intricate the integrin signaling processes are. Additionally, the RKKH peptide increases the binding of echo virus 1 to the recombinant α2I domain (Ivaska et al. 1999). Echo virus 1 was recently shown to favor the bent form of the α2β1 integrin (Jokinen et al. 2010). Chemically difficult cyclization of the peptide via terminal cysteine residues limits its usage as a tool in integrin research.

Studies done with the RKKH peptide showed that it is possible to use computer aided methods to understand the ligand binding in the case of the α2I domain. Amino acids proposed to be essential for the binding of the peptide can be used for the structure-based discovery of the small molecule ligands, and furthermore, the peptide can be used as a starting point when developing novel competitive small molecule ligands for the α2I domain.

2.3.2 Allosteric collagen inhibitors

Allosteric arylamide inhibitors were reported to inhibit the α2I domain-collagen binding with the recombinant proteins and in whole cells (Fig. 5) (Yin et al. 2006). These molecules were relatively large (molecular weight over 600 Da) and quite hydrophobic. The IC_{50} value of the most potent molecule in this series is 4.8 μM. Some structure-activity relationship speculations were presented, and in general, it was found that molecules containing aromatic hydrophobic fragments were the most effective inhibitors. The total charge carried by the ligands was not as important for effective binding. The binding site of these molecules was identified with NMR studies. Results suggested that the binding

site of these arylamides is the hydrophobic cleft on the side of the α2I domain instead of MIDAS. This cleft is situated in the area which has a critical role in the conformational changes upon the collagen recognition (Takagi et al. 2002, Yin et al. 2006). Amino acids Lys294, Lys301, Tyr311, Ser316, and Asp317 showed major changes in their chemical shifts when arylamide ligands were added. Additionally several other amino acids in the same region were affected. Lovastatin has been shown to bind at the similar site of the homologous αLI domain (Kallen et al. 1999).

Another group of allosteric regulators of the α2β1 were developed based on the inhibitors of the α4β1 and the αIIbβ3 integrins (Fig. 5). A prolyl-sulfonamide fragment and 2,3-diaminopropionic acid (DAP) were combined to discover the selective inhibitors of the α2β1 integrin (Choi et al. 2007, Miller et al. 2009). An approach where the inactive state of the integrin was stabilized via allostreric regulation was successfully employed by using molecules that bind to the βI domain (Xiong et al. 2001). The DAP derivatives did not inhibit the adhesion of the isolated I domain but were effective *in culture* when both α and β subunits were present. Thus, the inhibition mechanism was assumed to be allosteric. The DAP-based inhibitors showed to be very effective, having the IC_{50} value in the nanomolar range. Furthermore, ligands were selective over other β1 integrins. The effiency of the molecules was tested when the integrin was activated by the inside-out mechanism. This was done by platelet-activating mutations in the cytoplasmic tail of the α2 (O'Toole et al. 1994, Jung & Moroi 2000, Takagi et al. 2002, Wang et al. 2003). The best Pro-DAP analog can inhibit the collagen binding to the inside-out activated integrin, although a higher concentration of the inhibitor was required. The compounds did not have effect on the binding of the E318A mutant, thus, it was suggested that the Pro-DAP derivatives bind to the "closed" conformation of the I domain. Study demonstrated for the first time that a small molecule inhibitor of collagen-α2β1 integrin interaction show *in vivo* effiency in addition to the results obtained *in vitro* (Miller et al. 2009). Furthermore, the α2β1 was proven to be an excellent target for preventing pathological thrombus. Additionally, allosteric regulation of the collagen binding was suggested to be a suitable approach when the α2β1 integrin pharmaceuticals are sought.

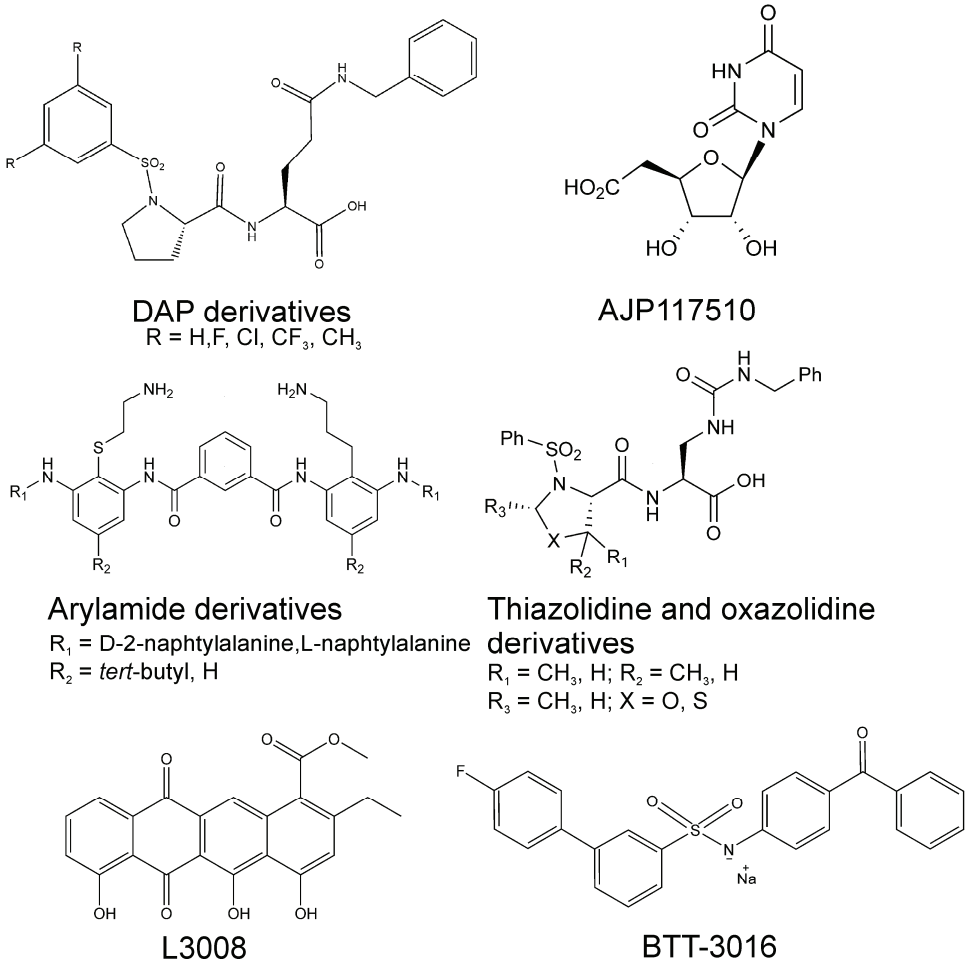


FIGURE 5 Collagen-α2β1 integrin inhibitors.

2.3.3 Competitive collagen inhibitors

Lack of natural small molecule ligands, which could be used as a starting point for the ligand development, is a problem when protein–protein interaction inhibitors are searched. Thus, the approaches for developing small molecule protein-protein interaction inhibitors are based on peptides, high-throughput screening or target-based computational methods (Wells & McClendon 2007, Zinzalla & Thurston 2009). Structure-based, also called target-based, discovery can be employed when the structure of target protein, as with the α2β1, is known (Käpylä et al. 2007, Nissinen et al. 2010). The competitive small molecule inhibitors that have been discovered presumably stabilize the closed, low-affinity conformation of the I domain, and therefore block the collagen binding into the α2β1 integrin (Käpylä et al. 2007, Nissinen et al. 2010).

Target-based pharmacophore model was utilized when designing the first competitive inhibitors of the collagen-α2 integrin interaction (Käpylä et al. 2007). These non-allosteric inhibitors were tetracyclic polyketides and extremely lipophilic, hence, not "drug-like" molecules (Fig. 5). However, the study represented that it is possible to rationally design molecules that will bind to the αI domain, and interfere with the collagen binding into it (Käpylä et al. 2007). Ligands were discovered based on the docking studies by using the crystal structure of the α2I domain in the closed conformation (PDB: 1AOX) as the target protein. Based on the structure of the MIDAS, the optimal ligands were assumed to fulfil the following structural criteria: (1) ability of Mg^{2+} coordination, (2) ability of forming interactions with amino acids of the αC helix (3) a suitable shape of molecules to fulfil the ligand binding groove of the MIDAS (Käpylä et al. 2007). Commercial molecular libraries were used to discover suitable molecules. Ligands that fulfilled the set structural criteria were tested in recombinant binding assays and in cell adhesion assays to explore their ability to inhibit the collagen-integrin interaction. As a result, the first three competitive α2–collagen interaction inhibitors were discovered. The most potent ligand had an IC_{50} value of 7,5–12 μM for the α2I domain–collagen interaction. Several other similar tetracyclic polyketides were tested as well, however, those compounds did not show inhibitor activity to the collagen-integrin interaction. This indicates that integrin binding is not a common property for this kind of molecules (Käpylä et al. 2007). Notable with these ligands is that they do not have a similar positive charge as the RKKH peptide (Fig. 5).

Recently, sulphonamides that inhibit collagen binding were found based on rational drug discovery approach (Nissinen et al. 2010). Molecules were built atom-by-atom to accomplish the criteria set by the pharmacophore based on the crystal structure of the closed conformation of the α2I domain (1AOX) and previous studies (Käpylä et al. 2007). One of the sulfonamides, BTT-3016 (Fig. 5), was presented as a new strategy for antithrombotic therapy (Nissinen et al. 2010). BTT-3016 was shown to block formation of thrombus in *in vitro* perfusion assay of human blood. Additionally, the mutations proved that the binding of BTT-3016 to the α2I domain is Y285 dependent as the modelling suggested. Furthermore, BTT-3016 was tested *in vivo* model in mice, wherein it could inhibit arterial thrombosis.

The selectivity of BTT-3016 has been widely explored (Nissinen et al. 2010). For example, it had only a minor effect on other collagen receptor integrins and no effect on the αI domain containing leucosyte integrin αLβ2. In addition to this, BTT-3016 showed no inhibition activity towards fibronectin and vitronectin receptor integrins that contain the β1 subunit, or to the ADP receptor P2Y12. Thus why, it is probable that the BTT-3016 analogues are selective for non-collagen receptor integrins (Nissinen et al. 2010). BTT-3016 proved, together with previous studies (Miller et al. 2009), that the α2β1 integrin is a potential target for the discovery of novel pharmaceuticals against thrombus. Additionally, this sulfonamide was proven to be a promising

scaffold for drug discovery. However, despite the detailed modelling results, the missing conclusions about structure-activity relationships of the sulfonamides restrain the drug development process.

3 AIMS OF THE STUDY

I Design and synthesize novel drug-like ligands that inhibit α2β1 integrin-collagen interaction. Aim was to improve the binding potency and solubility of the integrin ligands. Additionally, discover the structure-activity relationships of α2β1 integrin inhibitors.

II To develop small-molecule tools that can be used to study the integrin-collagen interaction with spectroscopic methods and in cellular level.

III Explore the physico-chemical properties of the synthesized molecules.

4 SUMMARY OF THE METHODS

Table 1 summarizes the methods used in this thesis. A more detailed description can be found from the original publications indicated by Roman numerals.

TABLE 1 Summary of the methods

Method	Publication
Molecular sketching and minimization	I, II, III, IV
Molecular docking	I, II, III, IV
Nuclear magnetic resonance spectroscopy (NMR)	I, II
Electrospray ionization mass spectrometry (ESI-MS)	I, II
Fluorescence spectroscopy	II
Absorption spectroscopy	II
Flash chromatography	I, II
Organic synthesis	I, II
Microwave assisted organic synthesis	I, II
X-ray crystallography	II
Cell culture	II
Ligand based molecular discovery	III, IV
Differential scanning fluorimetry (DSF)	IV

5 RESULTS AND DISCUSSION

5.1. Structure-activity relationships (I)

Previously reported studies of the RKKH peptide, allosteric regulators, and other α2 integrin ligands showed that computationally assisted methods (e.g. docking studies) can be successfully utilized in the αI domain ligand discovery. The effective pharmacophore model described earlier (Käpylä et al. 2007, Nissinen et al. 2010) was improved, and employed to carry out the first detailed structure-activity relationships study of the competitive α2β1 integrin ligands. A series of molecules based on BTT-3016 (Nissinen et al. 2010) was designed and explored to understand the important features of the α2β1 integrin inhibitors. Moreover, the aim was to improve the water solubility of the ligands, even tough some inhibitor potency would be lost. The pharmacophore model and docking simulations were found to predict the biological activity of the inhibitors quite well. However, the scoring functions of the utilized docking software were found to be inaccurate. Although the scoring functions were able to predict the binding potency of some molecules quite well, there were reliability problems with the scoring results. Similar reliability problems have been reported previously (Virtanen & Pentikäinen 2010). Thus, it was more convenient to perform predictions of ligand activity by using visualization.

The 3D structure of the α2β1 integrin I domain in the closed conformation (PDB code: 1AOX, Emsley et al. 1997) was used as a target protein in the ligand discovery. Based on the previous studies (Nissinen et al. 2010, Käpylä et al. 2007) plausible pharmacophore points were defined for the efficient binding into the MIDAS of the α2β1 integrin (Fig. 6): (a) the coordination with Mg^{2+}, (b) favorable hydrophobic contacts with Leu286 and Leu291, (c) a hydrogen bond with the hydroxyl group of Tyr285, (d) hydrogen bonds with the main-chain amino group of Glu256, and (e) with the main chain oxygen of Asp219.
First, molecule **4** was developed by using *de novo* ligand discovery and docking studies (Fig. 6A) (I). The docking results indicated that **4** could accomplish all features of the defined pharmacophore. The significance of the pharmacophore

points was then explored by changing the structure of the ligand one step at a time. To validate how the designed molecules can attain the interactions set in the pharmacophore, their binding capability was predicted by using docking studies.

The designed ligands that contain different benzophenone derivates or analogues were synthesized, and their biological activity was measured *in vitro*. The results from *in vitro* tests were subsequently compared to the outcomes of docking studies in order to evaluate the structure-activity relationships. The designed molecules were synthesized, characterized, and purified before biological testing. The sulfonamide synthesis procedure was optimized, ending up to the procedure summarized in Scheme 1. The workup was found to be efficient and producing high purity products without catalysts or chromatographic purification methods. Consequently, the utilized procedure was very convenient for this study although the yields were not high and varied a lot (20–70 %) (I, II). The usage of water in re-crystallization lowered the yield, since many of the synthesized molecules are soluble in water to some extent. In addition to the conventional synthetic methods, microwave assisted organic synthesis was successfully employed (II). The microwave synthesis of sulfonamides was done using small amount of pyridine as the solvent and raising the reaction temperature up to 90 °C. The purification was done in the same way as in conventional synthesis. This procedure gives quite similar yields in sulfonamide synthesis but it is much faster than conventional synthesis performed at room temperature (20 minutes compared to 12 hours, respectively) (I, II).

Sulfonyl esters were done using similar conventional procedure as with sulfonamides; only the base was changed to triethylamine (I). The purification was done by recrystallization from water-ethanol mixture using the procedure described in publications I and II. Methylated sulfonamides were synthesized in high yields using the procedure described in article I, and re-crystallized in the same way as primary sulfonamides and sulfonyl esters (I). All molecules were identified carefully using NMR, MS, elemental analysis, and whenever possible, X-ray crystallography.

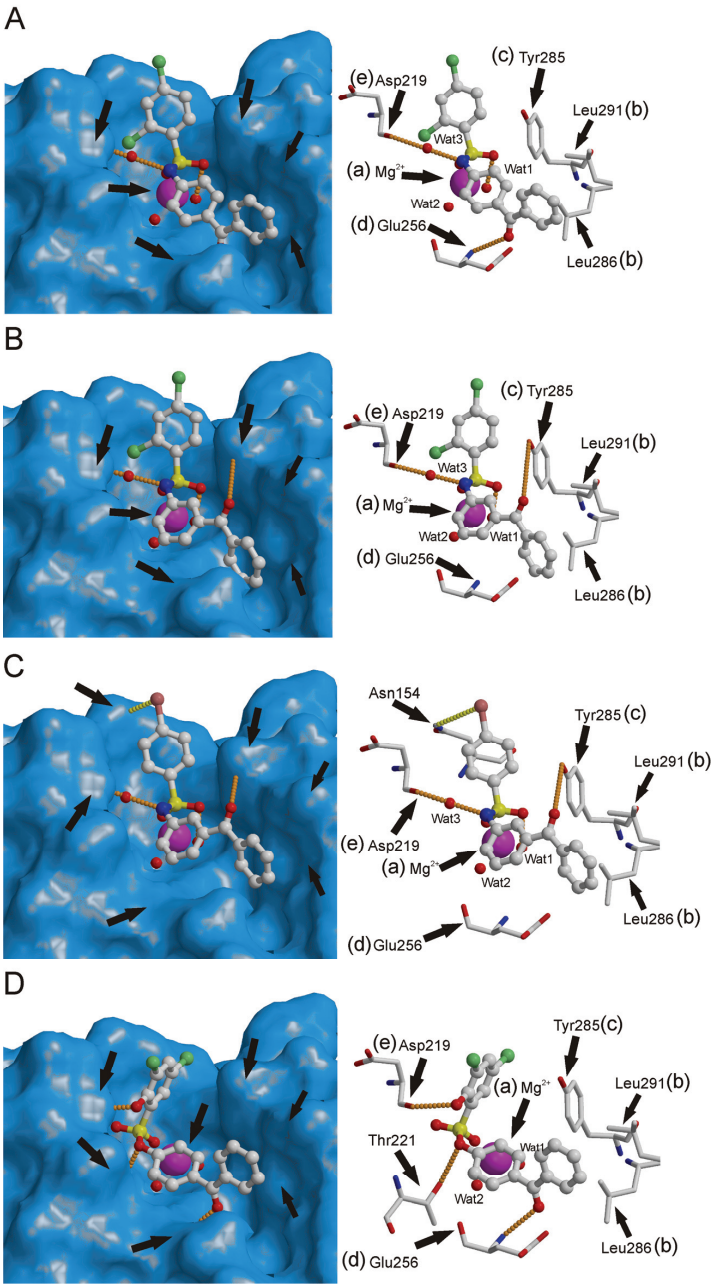
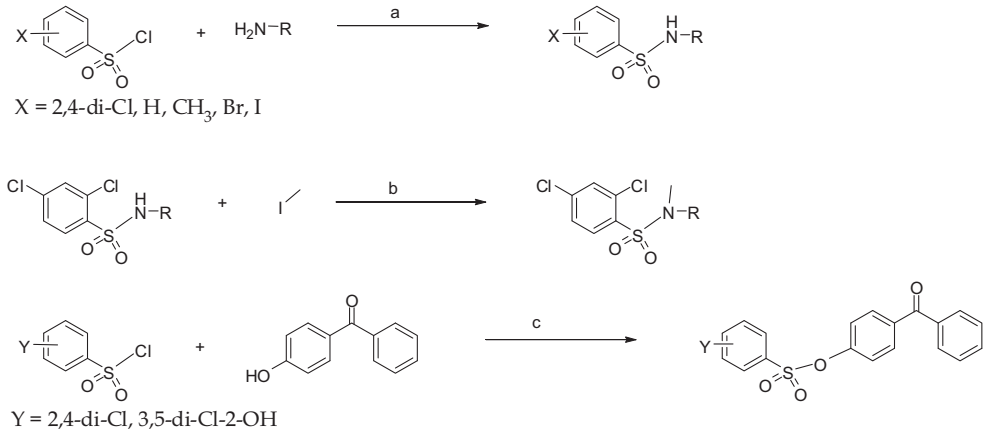


FIGURE 6 Key compounds of article I docked into the binding site. (A) Compound **4**, (B) Compound **5**, (C) Compound **17** and (D) Compound **21** docked into the MIDAS of the α2I domain. The hydrogen bonds are shown with orange dotted lines and favorable interaction between the bromine and NH group is shown with a yellow dotted line. Hydrogen atoms are omitted for clarity.

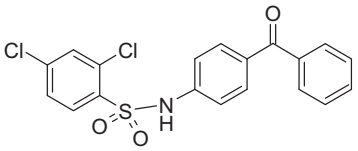
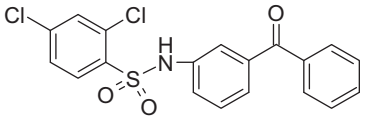
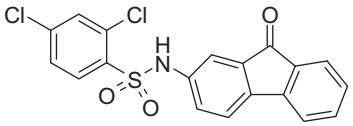
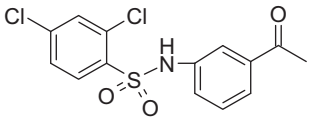
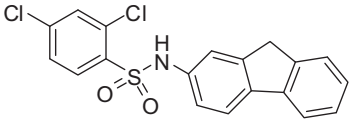


SCHEME 1 Synthesis a) pyridine, acetone, RT 12–16h b) K_2CO_3, DMF, RT 70h c) triethylamine, acetone, RT 12–14h.

5.1.1. Benzophenone moiety

Docking studies represented that the benzophenone moiety interacts with the side chains of Leu286 and Leu291, two hydrophobic amino acid residues demonstrated to be important for collagen binding (Compounds **4** and **5**, Table 2**,** Fig. 6A-B). Additionally, the ketone oxygen of benzophenone can form a hydrogen bond with the hydroxyl group of Tyr285 or with the main chain oxygen of Glu256. A more rigid molecule **6**, derived from fluorenone, showed no remarkable differences in the inhibitor activity compared to the benzophenone derivatives (EC_{50} 20 µM). Similarly to the previous studies (Käpylä et al. 2007), the hydrophobic contacts of the ligand with Leu286 and Leu291 were found to be crucial. Compound **7** that lacks a phenyl ring to interact with the aforementioned leucines, was ineffective as a collagen inhibitor (Table 2). In contrast, the hydrogen bond formed by the ketone oxygen with the main chain oxygen of Glu256 or with the side chain hydroxyl group of Tyr285, is not as critical. For example, compound **12** with fluorene moiety can inhibit collagen binding relatively well (EC_{50} 19 µM, Table 2). However, this ketone oxygen seems to affect the ligand selectivity between the α2 and α1 integrins (see chapter **5.1.5**).

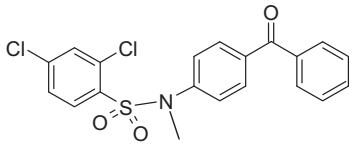
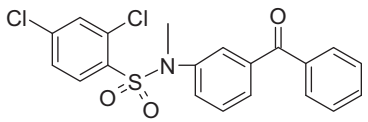
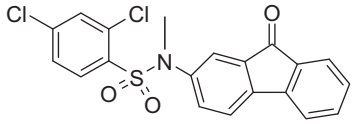
TABLE 2 Altering of the benzophenone moiety. Structures of the developed α2β1 integrin ligands, their biologically measured *in vitro* potencies. The IDs correspond to article I. Nd: Not determined; Modeling results obtained from the docking simulations by visualization: + indicates that the interaction with that group exists, +w indicates that the interaction is water-mediated; EC_{50} and inhibition measurements were repeated 2–4 times as a triplicate for each compound.

		Modeling	Experimental results	
ID	Compound	Mg^{2+} Leu286/Leu291 Glu256/Tyr285 Asp219	EC_{50} (μM)	Inhibition (%) 50μM±SEM Imax
4		+ + + +w	20	82±1 82
5		+ + + +w	17	69±4 69
6		+ + + +w	20	93±1 93
7		+ - + +w	Nd	0 Nd
12		+ + + +w	19	70±19 87

5.1.2. Sulfonamide core

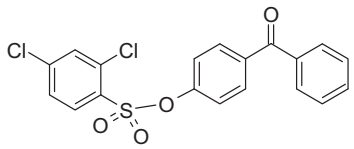
Docking of the sulfonamides suggested that they would form a water-mediated hydrogen bond to the main chain oxygen of Asp219. In order to test the importance of this hydrogen bond, several different sulfonamides and one sulfonic ester were designed (Table 3). The NH hydrogen of effective inhibitors **4**, **5**, and **6**, was replaced with a methyl group**,** thus, the hydrogen bond would be lost (Fig. 6, Table 3). The methyl group could fulfill the binding pocket more efficiently than hydrogen. On the other hand, the shape of the methylated ligands (**8–10**) changes to more "twisted" around the SO_2 group when compared to the non-methylated ones (**4–6**). Nevertheless, methylated sulfonamides are quite flexible and based on docking, fit well to the binding site. As a result, all molecules (**8–11**) lacking the NH group showed no inhibitor activity against collagen in the adhesion tests (Table 3). Consequently, these ligands proved that the hydrogen bond donor in this part of the α2β1 integrin ligand is essential for binding.

TABLE 3 Altering the sulfonamide core. The IDs correspond to article I. Nd: Not determined; Modeling results obtained from the docking simulations by visualization: + indicates that the interaction with that group exists.

ID	Compound	Modeling Mg^{2+} Leu286/Leu291 Glu256/Tyr285 Asp219	Experimental results EC_{50} (μM)	 Inhibition (%) 50μM±SEM Imax
8		-(+) - -(+) -	Nd	0 Nd
9		- - - -	Nd	0 Nd
10		- - - -	Nd	0 Nd

(continues)

TABLE 3 (continues)

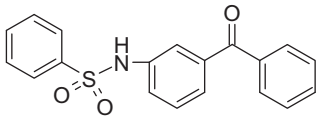
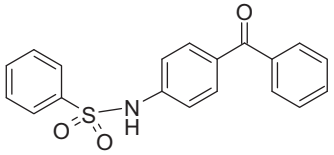
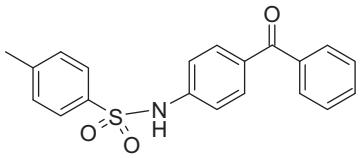
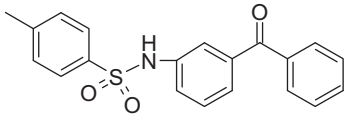
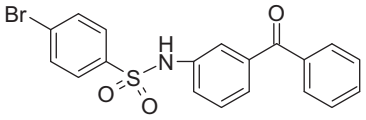
11		-(+) - -(+) -	Nd	0 Nd

5.1.3. Sulfonyl moiety

To study the structure activity relationships of the BTT-3016 analogues further, the sulfonyl fragment was systematically varied. Docking predicted that the chloro-substituent at the ortho position would be in the same direction as the hydrogen of the NH group, and moreover, that the ortho-chlorine could form a halogen bond to the main chain oxygen of Asp219 (Fig. 6A-B). Interestingly, similar conformation was observed in the crystal structures of these ligands. Bromo-substituted sulfonyl moiety showed promising results in docking studies and was found to be a very potent inhibitor, however, only with the 3-aminobenzophenone fragment (Table 4). The docking suggests that the bromine could interact with the side-chain carboxyl oxygen of Asp154 (Fig. 6C). Surprisingly, the docking results indicated that ligand with the 4-aminobenzophenone fragment (**18)** could not fulfill the criteria set in the pharmacophore model (Table 4). Furthermore, **18** showed only minor inhibitor activity *in vitro*. Even though the pharmacophore model is not perfect in the case of two bromo-substituted sulfonamides, the results correlate reasonably well when compared to the results achieved from the adhesion tests. The iodo-substituted ligands preferred 4-aminobenzophenone (Table 4). Docking predicted that this is caused by the bulkiness of iodine. Furthermore, the methyl group in the para position makes molecules considerably weaker inhibitors (Imax ~30 %, Table 4). Since methyl and bromine substituents have similar size, the interactions of the substituent of the sulfonyl moiety seem to be much more important than the shape.

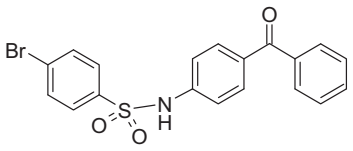
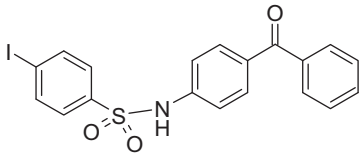
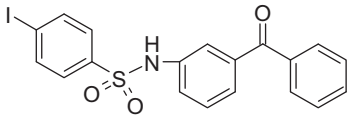
The pharmacophore model and docking studies could not completely clarify the implication of substituents of the sulfonyl fragment. Thus, some more molecules could be attached to series, such as 2-chloro and 4-chloro monosubstituted benzophenone derivatives. However, the structure-activity relationship study established that the properties of the sulfonyl fragment are crucial for effective binding. Even small changes in the sulfonyl-moiety substituents have great influence to the inhibitor activity compared to the effect of changes in the benzophenone fragment (Table 2 and 4).

TABLE 4 Varying of the sulfononyl fragment. The IDs correspond to article I. Nd: Not determined; Modeling results obtained from the docking simulations by visualization: + indicates that the interaction with that group exists; +w indicates that the interaction is water-mediated.

ID	Compound	Modeling Mg^{2+} Leu286/Leu291 Glu256/Tyr285 Asp219	Experimental results EC_{50} (μM)	 Inhibition (%) 50μM±SEM Imax
13		- - - -	Nd	4 Nd
14		- - - -	Nd	0 Nd
15		- - - -	Nd	35±5 35
16		- - - -	Nd	34±9 34
17		+ + + +w	16	92±1 96

(continues)

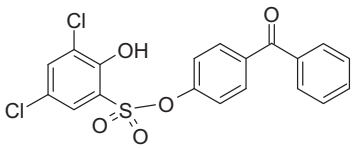
TABLE 4 (continues)

18		- - - -	Nd	34±4 34
19		+ + + +w	22	85±7 85
20		+ + + +w	40	72±15 72

5.1.4. Novel scaffold

Based on the results achieved from the set of sulfonamides, the next aim was to discover a novel collagen inhibitor scaffold (Fig. 6D, Table 5). Sulfonyl ester was found to be inactive (Table 3), however, the situation changed drastically when a hydrogen bond donor was added to the molecule in order to form a hydrogen with Asp219. The hydroxyl group at the ortho position can substitute the NH group of sulfonamides and form a direct hydrogen bond with Asp219 instead of a water-mediated. Additionally, based on docking studies, Ser214 can form a hydrogen bond with the hydroxyl group of **21**. This novel scaffold verifies that it is possible to discover more new α2β1 inhibitors by using the developed structure-activity relationship model and docking studies.

TABLE 5 The novel scaffold based on sulfonic ester.

ID	Compound	Modeling Mg^{2+} Leu286/Leu291 Glu256/Tyr285 Asp219 Ser 214	Experimental results EC_{50} (μM)	 Inhibition (%) 50μM±SEM Imax
21		+ + + + +	11	 86±4 95

5.1.5. Selectivity to α2 over α1

Designed molecules bind to the MIDAS of the I domain that is highly conserved in the α2 and α1 integrins. Furthermore, modeling suggests that the discovered ligands generally interact with the main chain atoms. Thus, it is expected that the molecules in this series do not achieve high selectivity (I). Nonetheless, some differences were discovered.

The main difference between the binding sites of the α2I and the α1I is that Tyr285 of the α2I is Ser284 in the α1I (Fig. 4). Despite the quite big difference in the size of these residues, only modest selectivity was achieved. Most likely this is due to the hydroxyl group in both side chains and the flexibility of the binding site. Fluorene derivative **12**, the only ligand lacking ketone oxygen, showed to be more potent against α1 compared to α2 (Table 2). It is presumable that the shape of the fluorene derivative favors the α1, and furthermore, the acidic CH_2 hydrogens can interact with the α1 better than the CO group of other ligands. Additionally, the 3-substitued aminobenzophenones showed slight selectivity to the α2 (I). Accordingly, the shape seems to be more important than a hydrogen bond acceptor in the corresponding area of the α2I ligand.

5.1.5. Unsuccessful scaffolds

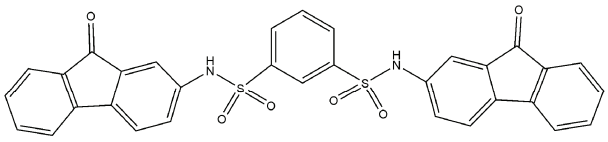
Some other, unpublished scaffolds were designed and synthesized without success. Amino acid derivatives gave promising docking results but were ineffective in the adhesion tests. However, when the acid was esterificated it showed some inhibitor activity. Interestingly, the previously discovered polyketide integrin ligands (Käpylä et al. 2007) are also esters. Presumably ligands based on free acids are too water soluble compared to their binding affinity. The spontaneous decomposition of amino-acid-based ligands also caused problems.

Overall, the structure-activity relationship study gave a good insight regarding the most important properties of sulfonamide ligands. The novel

ligands that were designed have considerably lower log P values compared to the lead molecule BTT-3016 (>5 of BTT-3016 compared to 3.4–4.1 of **4–21**, respectively). Consequently, the molecules of the SAR study provide valuable data that can be used in developing the lead molecule further.

5.2. Fluorescent probe (II)

The fluorenone moiety of the SAR study gave an idea to go further using the fluorescence properties of the ligands. The aim was to develop a molecule with better binding affinity to the α2β1 integrin compared to the previous molecules. CBL027 (Fig. 7) showed promising docking results, and moreover, it was a good inhibitor in adhesion tests (an EC_{50} value of 13 μM, II). In order to explore the binding site and the binding mode of CBL027, the inhibitor activity was tested with the Y285F mutant. The docking studies represent that CBL027 would lose one hydrogen bond when Tyr285 is mutated to phenylalanine, and as a result, some binding affinity would be lost (Fig. 8). The *in vitro* tests verified that CBL027 does not inhibit the binding of collagen to the α2I-Y285F mutant at 15 μM (Fig. 10A). However, CBL027 showed inhibitor activity when concentration was increased to 50 μM. Hence, the binding mode presented by docking studies appears to be reasonable.



CBL027

FIGURE 7 Structure of CBL027.

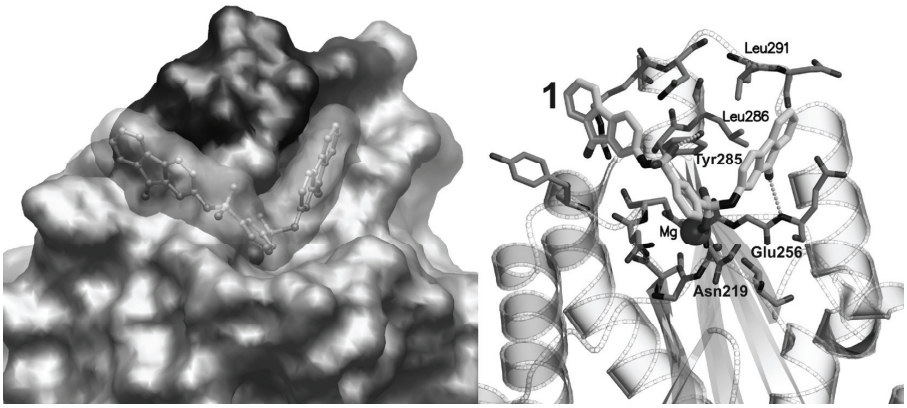


FIGURE 8 CBL027 docked into the MIDAS of the α2I domain.

Previous studies indicate that when fluorenone is shifted from polar solvent to more hydrophobic environment, the intensity of its fluorescence can increase (Fujii et al. 1996, Murphy et al. 1999). Most probably, a similar phenomenon can occur with the fluorenone derivative, CBL027. To test if the binding of CBL027 to protein changes the fluorescence or not, the CBL027 fluorescence was monitored when the recombinant α2I domain was added (Fig. 9B). Significant increase in the fluorescence intensity was observed. This phenomenon was only seen with the wild type α2I domain in the presence of Mg^{2+} ions, whereas the α2I C deletion mutant, the α2I in the absence of Mg^{2+} ions, or denatured protein showed no noteworthy effect on the CBL027 fluorescence (Fig 9C-D). Consequently, it is highly likely that CBL027 binds to the α2I domain in a Mg^{2+} ion dependent way, and is fluorescent when it forms a complex with the target protein.

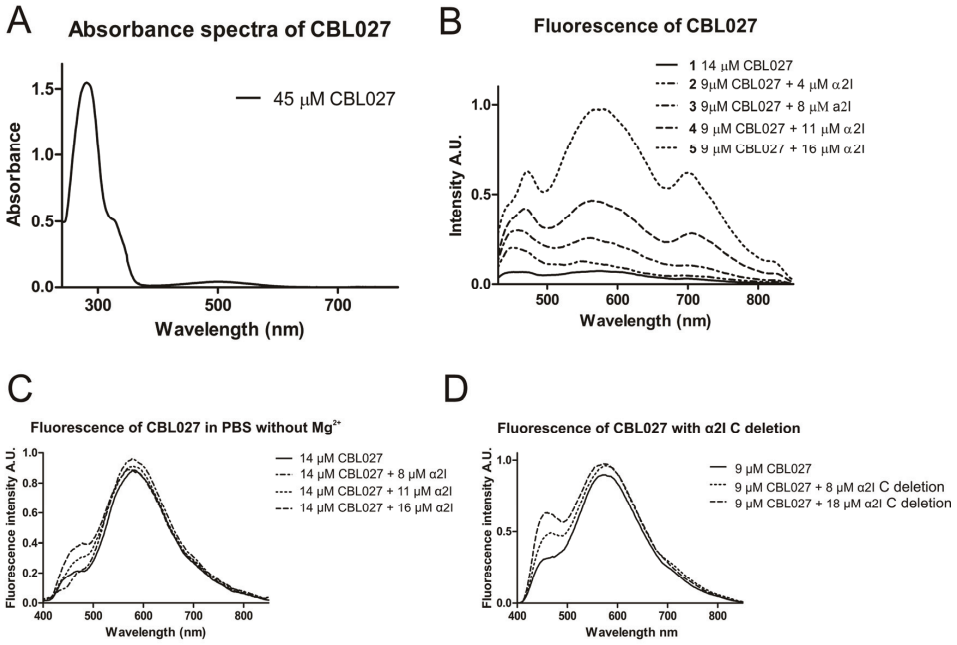


FIGURE 9 (A) Absorption spectra of CBL027 (B) Fluorescence spectra of CBL027 with the α2I domain and Mg^{2+} (C) Fluorescence spectra of CBL027 with the α2I domain without Mg^{2+} (D) Fluorescence spectra of CBL027 with the α2I C deletion.

Fluorescence-activated cell sorting (FACS) showed that cells overexpressing the α2β1 integrin can be labeled using CBL027 (Fig. 10B). The best wavelength to excitate CBL027 in FACS measurements was 430 nm (II), although the absorption spectra of CBL027 in PBS showed an absorption minimum at this wavelength and local absorption maxims of around 340 nm and 490 nm (Fig. 9A). Previous studies with fluorenone have shown that the absorption can move towards shorter wavelengths when molecule moves to a more

hydrophobic environment (Murphy et al. 1999). This result supports the results obtained from fluorescence spectroscopy.

Next, CBL027 was tested with L3008, an α2β1 integrin ligand described previously (Käpylä et al. 2007). The FACS studies showed that L3008 can decrease the amount of fluorescent cells, hence, binding of CBL027 (Fig. 10B). This reveals that CBL027 and L3008 have the same binding site. Therefore, CBL027 can be utilized when the binding site of novel integrin ligands is recognized. Moreover, CBL027 can be employed for developing a high-throughput screening method for competitive α2β1 integrin ligands.

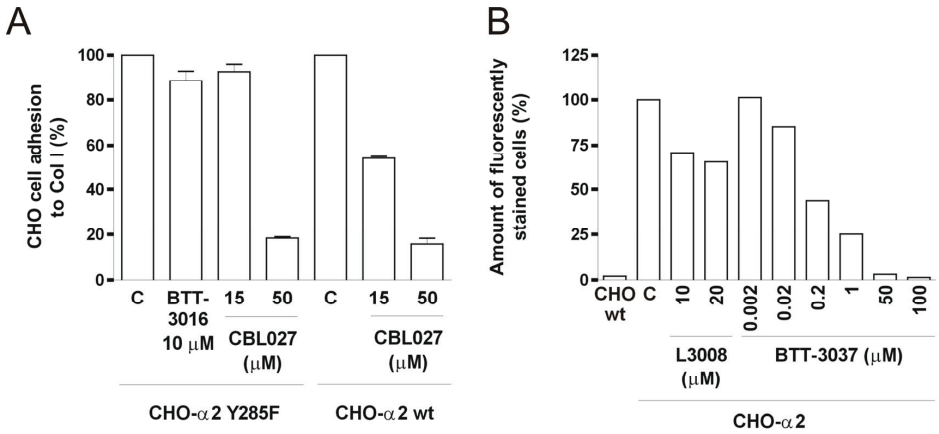


FIGURE 10 (A) CBL027 adhesion tests, BTT-3016 (Nissinen et al. 2010) was used as a reference (B) FACS analysis when CBL027 was competed with L3008 and BTT-3037.

5.3. Crystal structures

Previous studies have indicated that the conformations of the ligand crystal structures correlate relatively well with the structures of the ligands in complex with proteins (Brameld et al. 2008). Moreover, the packing of small molecules has been assumed to indicate the interactions that molecules form with proteins (Groom & Allen 2010).

We were able to solve several crystal structures of the discovered integrin ligands, including two polymorphs of CBL027 (Fig. 11A-B). These structures can be employed to compare the conformations of the docked ligands and their crystal structures. For example, as mentioned in the chapter **5.1.3**, chlorine in the ortho position points in the same direction as the NH hydrogen in both the docking results and in the crystal structures of **4** and **5** (Fig 6 A and B, Fig. 11 C and D). Furthermore, the crystal packing of **4** and **5** shows that NH of these ligands forms a hydrogen bond with carbonyl oxygen. Similarly to the crystal structures, the docking results of **4** and **5** suggest that ligands form a hydrogen

bond with the main chain oxygen of Asp219 (Fig. 6A and B, Fig. 11 C and D). In conclusion, the interactions seen in the crystal packing of the integrin ligands indicate that docking results are realistic in this point of view. Additionally, the crystal structures of CBL027 show that the "arms" formed by fluorenone fragments are quite flexible and can probably adopt several conformations.

The crystal structures of effective collagen inhibitors can be utilized in virtual screening from crystal databases in order to discover more α2β1 integrin ligands. So far, no α2β1 virtual screening method has been published. The crystal structures give a good starting point for developing an effective computer-aided screening method.

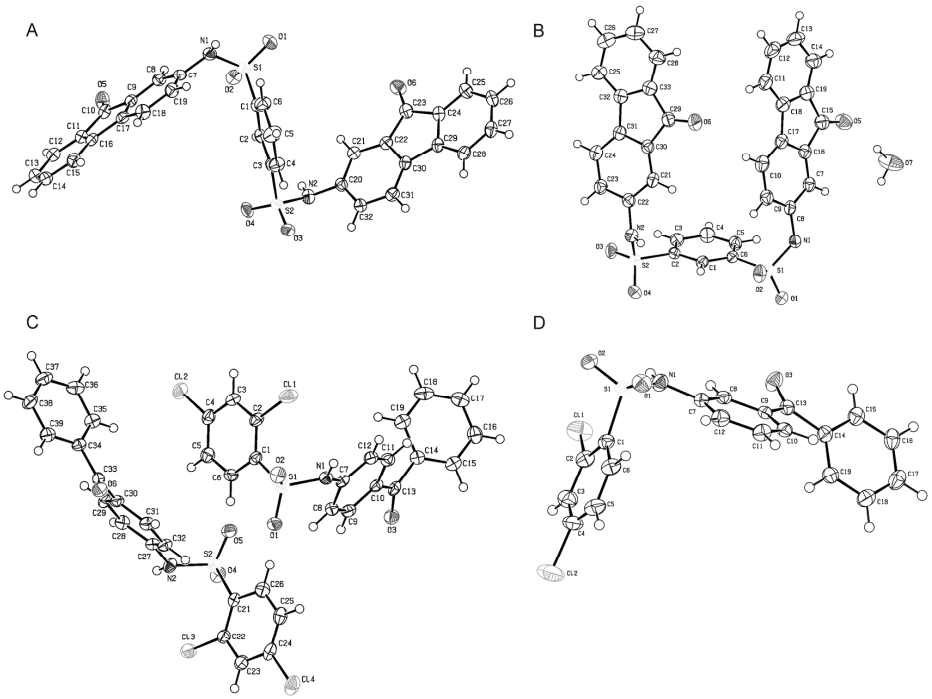


FIGURE 11 (A) Crystal structure of CBL027 (B) Crystal structure of CBL027 water solvate (C) Crystal structure of 4 (D) Crystal structure of 5.

5.4. Urea derivatives (III, IV)

Properties of the RKKH peptide and the sulfonamide ligands were combined by employing ligand-based drug discovery. Urea fragment was considered to be a suitable analog for the arginine of the RKKH peptide. As a result, urea-substituted sulfonamide derivatives were discovered (Fig. 12) (III, IV). Details about the synthesis of the urea derivatives are described in publication III. Urea substances did not fit the model used in the other α2 docking studies (I, II).

However, the urea moiety can be expected to interact with the acidic side chains of the α2I MIDAS. Some of these urea compounds were found to be very potent α2β1 integrin inhibitors. The best molecules have nanomolar EC_{50} values against collagen. However, the docking studies could not predict the binding mode of these ligands. Additionally, these ligands have similar features to the previously described allostreric inhibitors (Choi et al. 2007, Miller et al. 2009). Consequently, the new urea derivatives could function as allosteric regulators, and more experiments to solve the regulation mechanism were required.

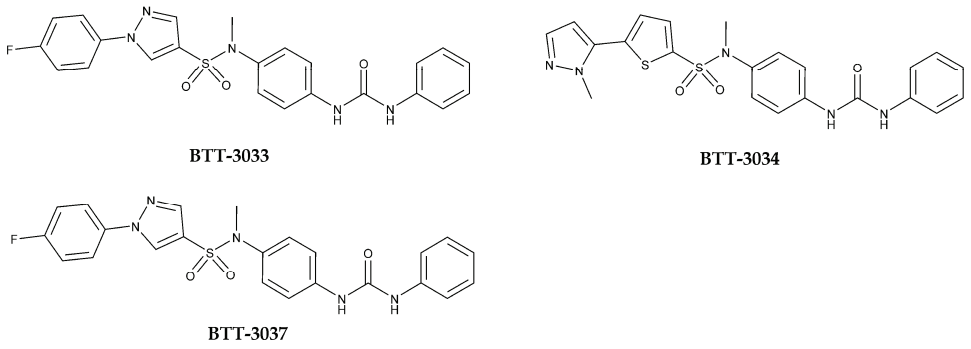


FIGURE 12 Integrin ligands developed in publications III and IV.

5.4.1. Identification of the binding site

The three most effective molecules, BTT-3033, BTT-3034, and BTT-3037 (Fig. 12), were explored in more detail using FACS and CBL027 to identify their binding mode. Two of them, BTT-3033 and BTT-3037, showed linear concentration dependent competitive binding with CBL027 (Fig. 10B). Surprisingly, BTT-3034, structurally similar with BTT-3033, competed only partially with CBL027 suggesting that it has an alternative binding site.

The inhibitor efficiency of BTT-3033 and BTT-3034 against collagen was tested with the CHO α2I-Y285F mutant. Similarly to CBL027, BTT-3033 does not inhibit collagen binding with Y285F mutant. In contrast, mutation had no effect on BTT-3034 inhibitor efficiency. BTT-3033 and BTT-3034 have sulfonyl and urea fragments that were described as essential features for tight binding of allosteric α2β1 integrin inhibitors based on the DAP scaffold (Miller et al. 2009). It might be possible that BTT-3034 has a similar binding mode to the earlier reported DAP derivatives.

The differential scanning fluorimetry (DSF; Pantoliano et al. 2001) was employed in order to test the binding of BTT-3033 and BTT-3034 to the α2I domain. Measurements showed that the binding of these two molecules reduces the thermal stability of the α2I domain. Furthermore, DSF showed that binding of these compounds into the α2I domain is Mg^{2+} dependent (IV). The DSF results together with cell-based experiments indicate that BTT-3033 could induce conformational changes in the α2I domain. To solve the detailed binding

mechanism, more experiments with the recombinant α2I domain and the developed small molecules should be done. For example, CD-spectroscopy and synchrotron radiation IR-spectroscopy are methods that could explain the possible conformational changes in the atomic level. Moreover, X-ray absorption spectroscopy could be used in order to explore the metal coordination of the ligand-α2I complex.

In article IV it was found that preactivation of α2β1 is not needed for collagen recognition under high shear. The activation of α2β1 was carried out by inside-out signaling and E318W mutation. Under flow, the adhesion was found to be most efficient with wild type integrins. Preactivation can even reduce the integrin mediated binding to collagen under shear stress. Furthermore, results obtained by using the xCelligence equipment suggest that the activation of α2β1 does not make the adhesion to collagen faster, but it can make it stronger at the final state. These results differ from the previous results obtained with leukocyte integrins. It appears that different integrins have completely distinct ligand recognition mechanisms. Some integrins, like α2β1, can bind ligands in the bent conformation, whereas others, like αLβ2, need to straighten up for ligand recognition.

BTT-3033 and BTT-3034 can both block CHO-α2β1 cells binding to collagen in static conditions with similar EC_{50} values. However, they seem to favor different activation states, thus, different conformations of the α2β1 integrin. Results indicate that BTT-3033 prefer the inactivated α2β1 and BTT-3034 the activated, standing α2β1 integrin. Additionally, BTT-3033 could prevent platelet adhesion to collagen in mouse whole blood under flow. In these conditions BTT-3034 could not inhibit platelet binding to collagen. Hence, molecules that favor the inactivated α2β1 integrin are more promising drug candidates for treatment of thrombus than ligands that bind to activated α2β1.

Results achieved with BTT-3033 support the results obtained with CBL027. The molecules have fully competitive binding and most likely have the same binding site. Furthermore, CBL027 was designed for closed conformation of the α2I domain, and BTT-3033 prefers the same conformation. However, in DSF studies BTT-3033 and CBL027 behave differently, as BTT-3033 reduces thermal stability of the α2I domain and CBL027 seems to stabilize it (unpublished results). Additionally, BTT-3033 does not fit in the pharmacophore model used with CBL027 and other sulfonamides (I, II). Consequently, BTT-3033 and CBL027 can have differences in the binding mechanism.

5.5. Small molecule integrin ligands – the future (I–IV)

Most likely the discovered integrin ligands work in three different ways: (1) the BTT-3016 based sulfonamide derivatives without a urea fragment are competitive collagen inhibitors that do not act as agonists; (2) the competitive urea derivates that inhibit collagen and act as a partial agonist of the integrin

α2β1; (3) allostreric α2β1 inhibitors. The molecules presented in this thesis give new insights into discovery of the α2β1 integrin ligands. Furthermore, the identified compounds can be used as tools in the drug development process, as well as when the integrin function is studied. It is possible or even presumable that the molecules with the binding mode similar to BTT-3016 cannot reach much higher binding affinity than BTT-3016. Molecules like BTT-3033 are much more effective collagen inhibitors, but they can act as agonists of the α2β1 integrin, i.e. can activate the integrin signaling process similarly to collagen. One future goal is to develop CBL027 further, for instance by combining fluorescent properties to urea derivatives. The results achieved in this thesis provide tools, information, and possibilities, but not the complete solution for the discovery of α2β1 pharmaceuticals. Different integrins, even the closely related ones, have versatile structure-function relationships. Thus, instead of generalizing results over all structurally similar integrins, the target integrin should be carefully explored in order to discover safe and effective drugs. It would be interesting to study the effect of the α2β1-collagen inhibitors on the binding of other natural α2β1 ligands, such as echo virus 1, decorin or laminins. This could provide valuable information about the inhibition mechanism of the ligands and the ligand recognition of the α2β1.

The discovered small molecule ligands offer new opportunities to crystallographic studies of the integrin αI domain. We have preliminary DSF results showing that CBL027 may increase the thermal stability of the α2I domain. It is possible that CBL027 can assist the crystallization of the α2I domain. In addition to this, the other ligands discovered here can also be used in protein crystallography to solve the first α2I domain-small molecule complex.

Sulfonamides presented in the structure-activity relationship study, as they are BTT-3016 analogs, most likely prevent the thrombus formation under flow. Furthermore, CBL027 competes with ligands that work in blood perfusion assays; therefore it could be utilized when a high-throughput screening method for α2β1 integrin ligands is developed. Moreover, BTT-3033 was shown to block the collagen-α2β1 integrin interaction under shear forces. Additionally, BTT-3033 seems to favor the inactivated conformation of the α2β1. The original approach to stabilize the inactive conformation of the α2I domain in order to block the collagen binding was proven to be accurate. However, BTT-3033 acts as an agonist of the α2β1 integrin. This is most likely an unwanted effect because agonism is found to be a problem in the drug development process (Cox et al. 2010). Nevertheless, this thesis presents various α2β1 integrin–collagen inhibitors and detailed information about their binding modes, and furthermore, several applications to design more of them. The results provide a great amount of information that can be used when α2β1 integrin pharmaceuticals are developed further. In conclusion, in this thesis work we have developed valuable tools for exploring the integrin function, and molecules that can be employed when new pharmaceuticals are screened, both virtually and *in vitro*.

6 CONCLUDING REMARKS

The main conclusions of this thesis are:

I. The structure-activity relationships of a series of α2β1 integrin inhibitors confirmed the following: (1) The utilized pharmacophore model is valid for designing molecules that block the binding of collagen I to the α2β1 integrin. (2) A hydrogen bond donor in the "middle" part of the ligand is critical for successful collagen inhibition. (3) Structural isomerism of the benzophenone fragment has only a minor effect on the binding potency. However, it has an effect on the selectivity between the integrin subtypes. (4) The keto-oxygen of the amino moiety has a role in the selectivity for α2 over α1. (5) The halogen substituent has greater impact on the efficient ligand binding than on changes in the benzophenone site.

II. Structurally similar α2β1–collagen inhibitors can have a completely different binding mechanism and binding site. The molecules discovered in this thesis most likely work in three different ways: (1) as competing antagonists (2) as competing inhibitors that are partial agonists (3) as allostreric inhibitors. The binding mode can be analyzed by using the fluorescent tool molecule developed during research conducted for this thesis. Moreover, the small molecules that were discovered can be used as tools for solving the unknown elements of the α2β1 integrin function as a collagen receptor.

III. The computational results correlate well with data achieved from the crystal structures of the α2β1 integrin ligands. Conformations of the docked ligands are reasonable according to the crystal structures. Furthermore, the intermolecular interactions of the ligands are comparable in the crystal structures and in the computational results.

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YHTEENVETO (RÉSUMÉ IN FINNISH)

α2β1-integriiniligandien suunnittelu; lääkeaihioita ja työkaluja syövän ja veritulpan hoitoon

Integriinit ovat solun pinnan proteiineja, joiden avulla yksittäiset solut kiinnittyvät soluväliaineeseen. Integriinit myös välittävät signaaleja solun sisältä ulos ja päinvastoin. Ihmiseltä on löydetty 24 erilaista integriiniä, joista neljä sitoutuu pääasiassa kollageeniin. Solujen ja kollageenien välinen vuorovaikutus on osallisena myös eräissä tautimekanismeissa, kuten veritulpan syntymisessä ja syöpäsolujen leviämisessä. Integriinisalpaajista toivotaan uutta hoitomuotoa verisuonitukosten sekä syövän hoitoon ja ennaltaehkäisyyn. Viimeaikaiset tutkimukset ovatkin osoittaneet, että α2β1-integriini on potentiaalinen lääkekehityksen kohdeproteiini. Tästä huolimatta vain muutamia α2β1-integriiniin vaikuttavia lääkeaihioita on pystytty kehittämään. Osaltaan tämä johtuu siitä, ettei α2β1-integriinin rakenteen ja toiminnan välistä yhteyttä ole pystytty selvittämään tarkasti.

Tässä työssä kehitettiin tietokoneavusteisesti useita α2β1-ligandeja. Aluksi selvitettiin α2β1–kollageeni-inhibiittoreiden rakenne-aktiivisuussuhteita suunnittelemalla 17 uutta integriiniligandia käyttäen farmakoforimallia, joka perustuu aiemmin ratkaistuun α2I-domeenin kiderakenteeseen. Tämän mallin avulla löydetyt molekyylit pystyivät solukokeissa estämään α2β1-integriinin kiinnittymisen kollageeniin. Tutkimus myös paljasti tehokkaiden integriinisalpaajien tärkeimpiä ominaisuuksia. Näiden tulosten pohjalta pystyttiin kehittämään fluoresoiva työkalumolekyyli, jota voidaan käyttää selvitettäessä α2β1-integriinin toimintaa sekä kehitettäessä suurtehoseulontamenetelmiä. Lisäksi tällä työkalumolekyylillä pystytään tutkimaan muiden α2β1-ligandien sitoutumispaikkaa.

Farmakoforimallin lisäksi integriinisalpaajia kehitettiin myös ligandipohjaisesti hyödyntäen aiemmin löydettyjä α2β1-integriiniin sitoutuvia pienmolekyylejä ja peptidejä. Osa näistä ureapohjaisista molekyyleistä osoittautui erittäin tehokkaiksi ja niistä on vireillä patenttihakemus. Näillä molekyyleillä saadaan myös uutta tietoa α2β1-integriinin toiminnasta.

Useiden kehitettyjen integriiniligandien kiderakenteet pystyttiin ratkaisemaan. Pienmolekyylien kiderakenteita voidaan käyttää kehitettäessä tehokkaita virtuaaliseulontamenetelmiä, joiden avulla voidaan löytää yhä uusia α2β1-kollageeni-inhibiittoreita. Rakenteiden avulla voitiin varmentaa oikeiksi laskennallisilla menetelmillä saadut tulokset.

Tässä väitöskirjatutkimuksessa ratkaistiin α2β1-ligandien tärkeimpiä ominaisuuksia sekä pystyttiin osoittamaan käytetyn farmakoforimallin paikkansapitävyys. Nämä tulokset mahdollistavat α2β1-integriiniin kohdistettujen lääkeaihioiden jatkokehityksen. Tutkimuksessa pystyttiin kehittämään työkaluja, joiden avulla voidaan tutkia solujen vuorovaikutusta kollageenin kanssa, veritulpan muodostumista sekä syövän etäispesäkkeiden syntymekanismia. Koko-

naisuutena nämä tulokset antavat paljon mahdollisuuksia turvallisten ja tehokkaiden α2β1-integriinilääkkeiden kehitykseen.

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BIOLOGICAL RESEARCH REPORTS FROM THE UNIVERSITY OF JYVÄSKYLÄ

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