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Polythiazole linkers as functional rigid connectors: a new RGD cyclopeptide with enhanced integrin selectivity†

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Polythiazole amino acids clasp linear peptides to generate cyclic derivatives, however, the resulting species are not merely stapled peptides but bear a complex heterocyclic moiety displaying its intrinsic set of interactions. As a proof of concept, a bisthiazole moiety has been grafted onto an RGD sequence to deliver a new cilengitide analogue with improved integrin selectivity and remarkable *in vivo* antiangiogenic activity.

Introduction

In recent years, the pharmaceutical industry has undergone a profound transformation. In the period 2009–2012, the US FDA approved the release of only 116 new therapeutics; of these, 91 were new chemical entities and 25 were new biologics.[1] An analysis of the chemical entities by chemical species reveals that the so-called "small molecules" have been losing ground over the past few decades, since they represent only 48% of total FDA approvals, as compared with the aforementioned biologics (21%). Interestingly, peptides account for 14 new therapeutic agents (12%).[2] This change in scenario reflects the general acceptance that peptides may be used as drugs. The main drawbacks of peptides as drugs are their relatively high flexibility, lack of stability, and poor pharmacokinetic properties when compared with small molecules. Over the years, peptide chemists have addressed these issues in two main ways, namely by cyclization[3] or by the introduction of non-natural amino acid-like moieties, such as *N*-alkyl-,[4] rigidified or chemically modified amino acids[5] and β-amino acids.[6] Cyclization was first implemented *via* a homodetic linkage through a "head-to-tail"—as illustrated by cilengitide—[7] or "side chain-to-side chain" (Lys-Asp/Glu) topology. Later, Gilon and coworkers introduced the backbone-to-backbone cyclization concept to preserve the identity of the side chains.[8] Although disulfide bridges frequently appear in many biologically active peptides and efficiently contribute to their stabilization, their redox properties jeopardize their introduction in the *de novo* design of cyclic peptides. In this regard, several authors have proposed the replacement of the S–S bonds with dicarba analogs through metal-catalyzed ring-closing olefin metathesis.[9] This approach has led to the "stapled" peptide concept introduced by Verdine,[10] where a flexible hydrocarbon motif is introduced. A further advance has been the development of cyclic peptides through Sharpless click chemistry,[11] which incorporate a triazole ring on the cyclization branch.

Additionally, heterocycles are a recurring motif in the medicinal chemistry toolbox,[12] and their presence can overcome some of the problems associated with the use of peptides as drugs. In this context, the "heterocycle-connected" cyclic peptide concept is described herein. In a broad sense, these rigid connectors can be regarded as "functional staples": modular and synthetically amenable structural units based on the suitable intercalation of (poly)thiazole moieties (or other groups) by amide formation, which enables fitting of the appropriate length, introduces rigidity, and improves drug likeness for the development of a new class of cyclic peptide. Furthermore, they retain almost all of the features of classical staples (conformational restriction and metabolic stability), leading to new structural features that improve the peptide–target interaction. On the other hand, this approach requires more specific synthetic work (short sequences to prepare the

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thioazole amino acids) and, although not as direct as the initial stapling techniques, is quite straightforward.

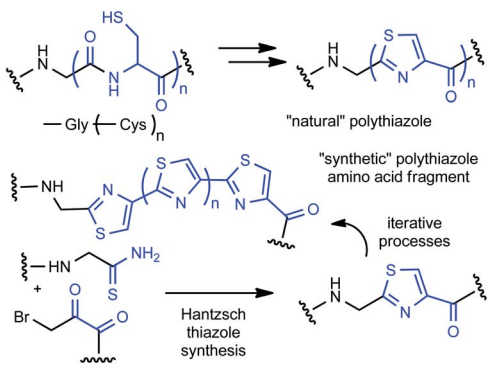
Results and discussion

Natural biosynthetic thiazoles (2,4-disubstituted) can be considered derivatives of Cys-containing peptides. Thiazole peptides are formed through a cyclodehydration step followed by an oxidation step, thus rendering their synthesis extremely challenging. A synthetic alternative involves the use of pre-synthesized (poly)thiazole amino acids in a standard solid phase peptide synthesis. The thiazole precursors, featuring a 2,4-disubstitution pattern, can be prepared through a Hantzsch thiazole synthesis by the condensation reaction between thioamides and α-haloketones. (Scheme 1). Incidentally, oxazoles are structurally isosteric to thiazoles, and they would be very likely to behave in a similar way. However, although they are biosynthesized through related cyclodehydration mechanisms, they require harsher reaction conditions for chemical synthesis and were not considered in this work.

The corresponding synthetic (poly)thiazole-containing amino acids can be coupled to the *N*- and *C*-terminal sites of an active peptide motif to prepare a head-to-tail cyclic peptide or to side chains of Lys and Asp/Glu to give side chain-to-side chain derivatives (or a combination of both). As a proof of concept, analogs of the integrin antagonist cilengitide (cyclo[R-G-D-f-(NMe)V]) of different sizes containing thiazole motifs have been synthesized. Cilengitide is actually in advanced clinical phases for use against glioblastoma and other cancers as an anti-angiogenic agent, either alone or in combination with radio/chemotherapy. Although so far no significant efficacy has been demonstrated in the concluded trials, preliminary data suggest that this compound could become a useful antitumoral drug.[7] Angiogenesis involves a complex sequence of biological events that lead to the formation of blood vessels. Tumor growth occurs by cell division and is limited by the availability of nutrients. To go beyond this point, tumors promote angiogenesis in order to irrigate the new cell mass and, in this way, maintain expansion. Therefore, antiangiogenesis strategies are considered to be highly attractive ways to treat cancer.[13]

The interaction between the extracellular matrix and membrane proteins controls cell adhesion, tissue formation and transmembrane signaling. In this context, integrins comprise a family of transmembrane heterodimeric receptors and have emerged as a promising target because of their prominent role in many biological functions.[14] A subset of integrins specifically recognizes the tripeptide sequence Arg-Gly-Asp (RGD) in their ligands. In particular, the $\alpha_v\beta_3$, $\alpha_v\beta_5$ and $\alpha_5\beta_1$ subfamilies participate in tumor metastasis and tumor-induced angiogenesis. Furthermore, endothelial cell survival and proliferation are strongly dependent on the adhesive activity of $\alpha_v\beta_3$ and $\alpha_v\beta_5$. Thus, several integrin antagonists have been designed for therapeutic purposes, including those with an RGD scaffold as well as others that are structurally unrelated.[4,15] After the pioneering studies of Kessler and coworkers, which culminated with the development of cilengitide,[7] the rational design of antagonists has pursued the control of the three-dimensional features of the RGD sequence in order to improve selectivities and pharmacokinetic profiles and to allow for the development of strategies for drug delivery and bioimaging.[16] There are a considerable number of distinct integrins and their different cellular roles determine several events, including among them the blockade of tumor progression in some cancer cell lines. Furthermore, the often complex binding profiles of most integrin antagonists severely restricts the therapeutic and bioimaging applications of such compounds. Therefore, although potent antagonists have been developed, more selective agents are still needed, both as probes to study the fundamental biology of integrins and as theragnostic agents. Given the relevant presence of thiazole nuclei in several bioactive natural products,[17] we included these structures in an RGD cyclopeptide in order to determine their antiangiogenic activity. In particular, we synthesized and characterized compounds **1–3** (Fig. 1), which bear 1, 2 or 3 thiazole units in the tether, and were likely to fit the specificity determining loop (SDL)[18] and display a range of affinities for the integrin receptors.[19]

To this end, we envisioned the synthesis of the parent thiazole amino acids, which were prepared through a unified approach involving an iterative protocol based on the Hantzsch thiazole reaction (Scheme 2).[20] In this way, treatment of *N*-benzyloxycarbonylglycine with isobutyl chloroformate (IBCF), in the presence of *N*-methylmorpholine (NMM) as a base, followed by the reaction with aqueous NH_3 afforded amide **4**. This compound was transformed into the corresponding thioamide **5** by interaction with the Lawesson reagent (L.R.), and its



Scheme 1 Biosynthetic and chemical access to thiazole peptides.

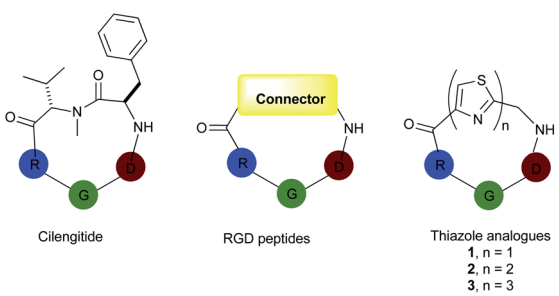
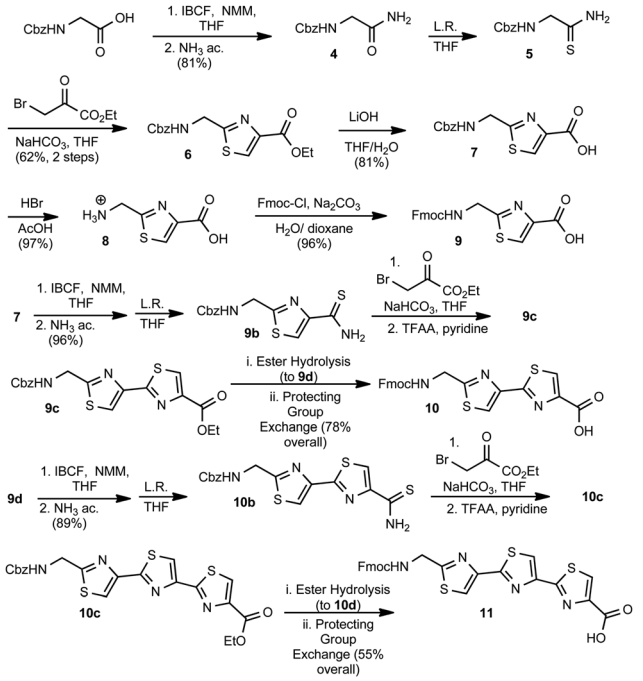
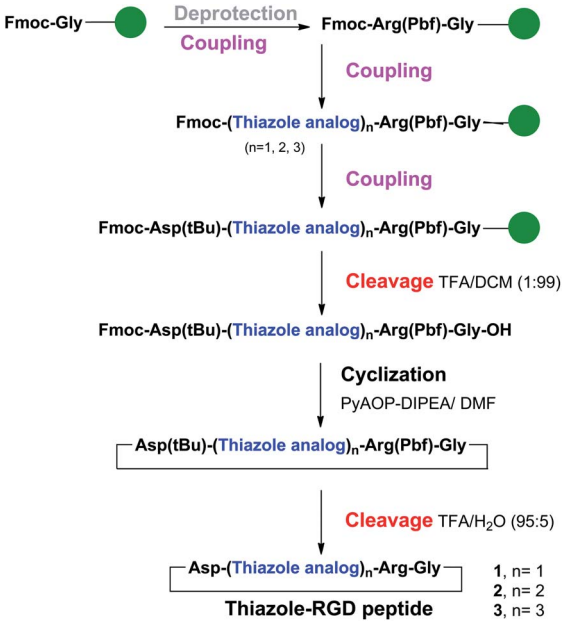


Fig. 1 Cilengitide, RGD peptides and thiazole analogs **1–3**.



Scheme 2 Synthesis of the thiazole-based amino acids **9**–**11**.



Scheme 3 Synthesis of the thiazole-based peptides **1**–**3**.

reaction with ethyl bromopyruvate led to thiazole **6**. Subsequent hydrolysis of the ester group afforded carboxylic acid **7**. Deprotection of the *N*-Cbz group and introduction of the Fmoc substituent yielded amino acid **9**, which was suitable for coupling by a subsequent solid-phase methodology using Fmoc/*t*Bu chemistry. The bis- and tris-thiazole amino acids **10** and **11** were prepared analogously (Scheme 2).

The linear precursors of the cyclopeptides were synthesized by standard solid-phase methodology using 2-chlorotrityl resin and Fmoc/*t*Bu chemistry followed by a final solution phase cyclization. The protecting groups of the side chains of Asp and Arg, *t*Bu and Pbf, respectively, were stable to the trifluoroacetic acid (TFA) concentrations used for the peptide release from the 2-chlorotrityl chloride resin (Scheme 3). The cyclization reactions between the amino group of Asp and the carbonyl group of Gly were performed in solution using PyAOP as a coupling agent, furnishing the protected cyclic peptides. The final deprotections to remove the *t*Bu and Pbf groups were carried out without triisopropylsilane (TIS) as a scavenger to prevent the reduction of the thiazole rings. The corresponding cyclopeptides with thiazoline residues were detected in experiments with TIS (M + 2 peaks were found in the HPLC-MS analysis), thus suggesting an incompatibility between the thiazoles (or related imine-containing structures) in acidic media and silicon hydrides. Thus, the optimized conditions involved treatment with TFA/H_2O (95 : 5) (Scheme 3). In this way, a scalable synthesis of thiazolopeptides **1**,[21] **2** and **3** was ensured.

Although direct binding assays with purified integrins are the most direct and specific method to determine the antagonists' selectivities,[4,7c,15e] we have determined IC_{50} values from cell-based competition experiments in order to facilitate the direct comparison of the present data with the activities reported in previous studies. On the other hand, these tests (Fig. 2) provide a robust demonstration of the real activity of these compounds under physiological conditions, which is more reliable for prospective therapeutic applications. The biological assays were performed with the following cell lines: DAOY (human medulloblastoma cells, expressing both $\alpha_v\beta_3$ and $\alpha_v\beta_5$ members of the α_v-integrin family), HT29 (human colon adeno-carcinoma, expressing both $\alpha_v\beta_5$ and $\alpha_v\beta_6$ integrins), and HUVEC (human umbilical vein endothelial cells as the primary cell culture, expressing both $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins). As integrin ligands, in these experiments we used vitronectin (VN), fibrinogen (FB), fibronectin (FN) and collagen (COL). The experimental protocol was set up *via* ligand coating followed by analysis of the compound's effect on cell adhesion to the ligand. Briefly, matrix proteins were coated onto 96-well plates, the wells were blocked with bovine serum albumin (BSA) and serially diluted solutions of compounds were added followed by the cells in the cell culture medium. After 1 hour at 37 °C, the non-

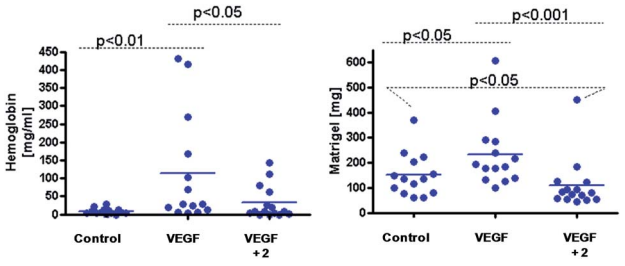


Fig. 2 Inhibition of angiogenesis by peptide **2** in the *in vivo* experiments by the Matrigel plug assay. Hemoglobin contents of control vehicle, VEGF and VEGF + peptide **2** (2 μg mL^{-1}, left graph) and plug weight of the same samples (right graph). Mean and statistical significances are shown.

adherent cells were washed away and the amount of attached cells remaining was measured using the hexominidase enzymatic assay. The inhibition of attachment was calculated using wells with no added compounds as a reference, and the results are summarized in the table as IC_{50} values in μM concentrations for compounds **1–3** and cilengitide (**A**; used as the reference control), tested in HUVEC, DAOY and HT-29 cells (Table 1).

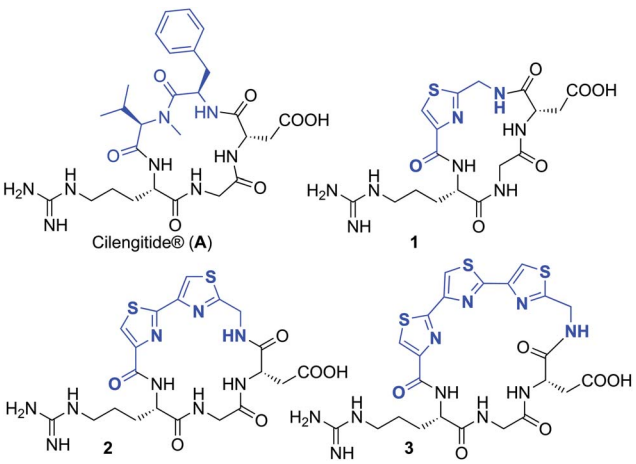
In the experiments using VN as the integrin ligand, the HUVEC and DAOY cells used $\alpha_v\beta_3$ and $\alpha_v\beta_5$ to bind to this protein, whereas HT-29 cells used only $\alpha_v\beta_5$. Complementarily, in the tests in which FB was the integrin ligand, HUVEC and DAOY cells used $\alpha_v\beta_3$ to bind to it. Experiments involving FN and COL were run to check that the new peptides did not block the cell adhesion mediated by other integrins (the β_1 subfamily). The results indicated that compounds **2** and **3** showed satisfactory inhibitory activity in the HUVEC/DAOY cells for VN, but they were less potent than cilengitide. However, these peptides displayed potencies similar to cilengitide with respect to blocking $\alpha_v\beta_3$-mediated cell adhesion with FB. This observation suggests that **2** and **3** show different affinities to $\alpha_v\beta_3$ and $\alpha_v\beta_5$. Furthermore, the results obtained for the HT-29–VN system showed that these cyclopeptides do not exert significant inhibitory activity against $\alpha_v\beta_5$, thereby indicating that, unlike cilengitide, they are extraordinarily specific for $\alpha_v\beta_3$. Compound **2** displayed interesting potency and selectivity, even compared with cilengitide, against several cell lines (Table 1). These data reveal that the bisthiazole cyclopeptide has the correct structure to accommodate an extended RGD sequence in the cleft, similar to that of cilengitide.

To further confirm the activity of cyclopeptide **2**, the capacity of this compound to inhibit angiogenesis was explored using *in vivo* assays in conjunction with both positive (VEGF) and negative controls. The results obtained in the matrigel plug assay[22] revealed an antiangiogenic effect of **2** ($p < 0.05$) *in vivo*, showing a lower level of haemoglobin in the treated plug than in the VEGF control (Fig. 2). Moreover, the average weight of the plug was also significantly lower than in the positive (VEGF) and negative controls. These data unequivocally show that this compound efficiently represses angiogenesis in living animals, thereby demonstrating its capacity as a potential therapeutic agent.

To rationalize these findings, compound **2** was docked in the binding site of $\alpha_v\beta_3$ and $\alpha_v\beta_5$, and the structural integrity of the binding mode was further checked using molecular dynamics (MD) simulations. Similar studies were performed for cilengitide (**A**), which was used as a reference system. To this end, the structure of **2** was determined by combining NMR-based restraints with MD simulations of the cyclic compound, and a 3D model of $\alpha_v\beta_5$ was developed by homology modeling (see details in the ESI†).

The docking of **A** to the binding site of $\alpha_v\beta_3$ reproduced the binding mode found in the X-ray structure of the complex between **A** and $\alpha_v\beta_3$ (PDB ID: 1L5G), which was stable throughout the 50 ns MD simulation (the positional root mean square deviation (rmsd) of the modeled backbone of **A** *versus* the X-ray structure was 0.8 Å). This can be primarily attributed to the stabilizing contributions from the Asp and Arg residues in the RGD stretch: the former interacts with the metal-ion-dependent adhesion site (MIDAS; in β_3), and the latter forms a

Table 1 Activities (cell adhesion inhibition) of compounds **1–3** in the cell systems, expressed as IC_{50} in μM



VN or FB coating [μg mL^{-1}]	Cell lines (integrin profile)	1	2	3	A (cilengitide)
VN [0.75]	HUVEC ($\alpha_v\beta_3/\alpha_v\beta_5$)	31.13	7.46	63.80	0.56
VN [0.5]	DAOY ($\alpha_v\beta_3/\alpha_v\beta_5$)	ND	5.71	6.80	0.068
VN [4.0]	HT-29 ($\alpha_v\beta_5$)	ND	53.16	59.05	2.24
FB [4.0]	HUVEC ($\alpha_v\beta_3$)	368.4	4.95	33.15	4.03
FB [5.0]	DAOY ($\alpha_v\beta_3$)	ND	**0.082**	1.86	0.059

salt bridge with D248 and a cation–π interaction with Tyr208 (both in α_v), respectively (Fig. 3A). The net effect is to keep cilengitide tightly bound in the binding pocket. Importantly, the results obtained for the binding to $\alpha_v\beta_5$ showed that **A** assumed a binding mode that matched the binding to $\alpha_v\beta_3$ (Fig. 3B). Analysis of the pairwise interactions with the residues in the binding site also points out that the binding of **A** to both $\alpha_v\beta_3$ and $\alpha_v\beta_5$ is assisted by the van der Waals contacts formed by Phe, mainly with Tyr148 in β_3 and Leu148 in β_5, and the hydrogen bond between the NH group of Asp with the carbonyl group of Arg242 in β_3 and Arg243 in β_5.

Compound **2** bound to $\alpha_v\beta_3$ and $\alpha_v\beta_5$ retained the interactions with both the metal cation at MIDAS and Asp248 (α_v). Nevertheless, while **2** superposes well with the structure of cilengitide upon binding to $\alpha_v\beta_3$, a significant relocation of the cyclopeptide backbone is observed upon binding to $\alpha_v\beta_5$

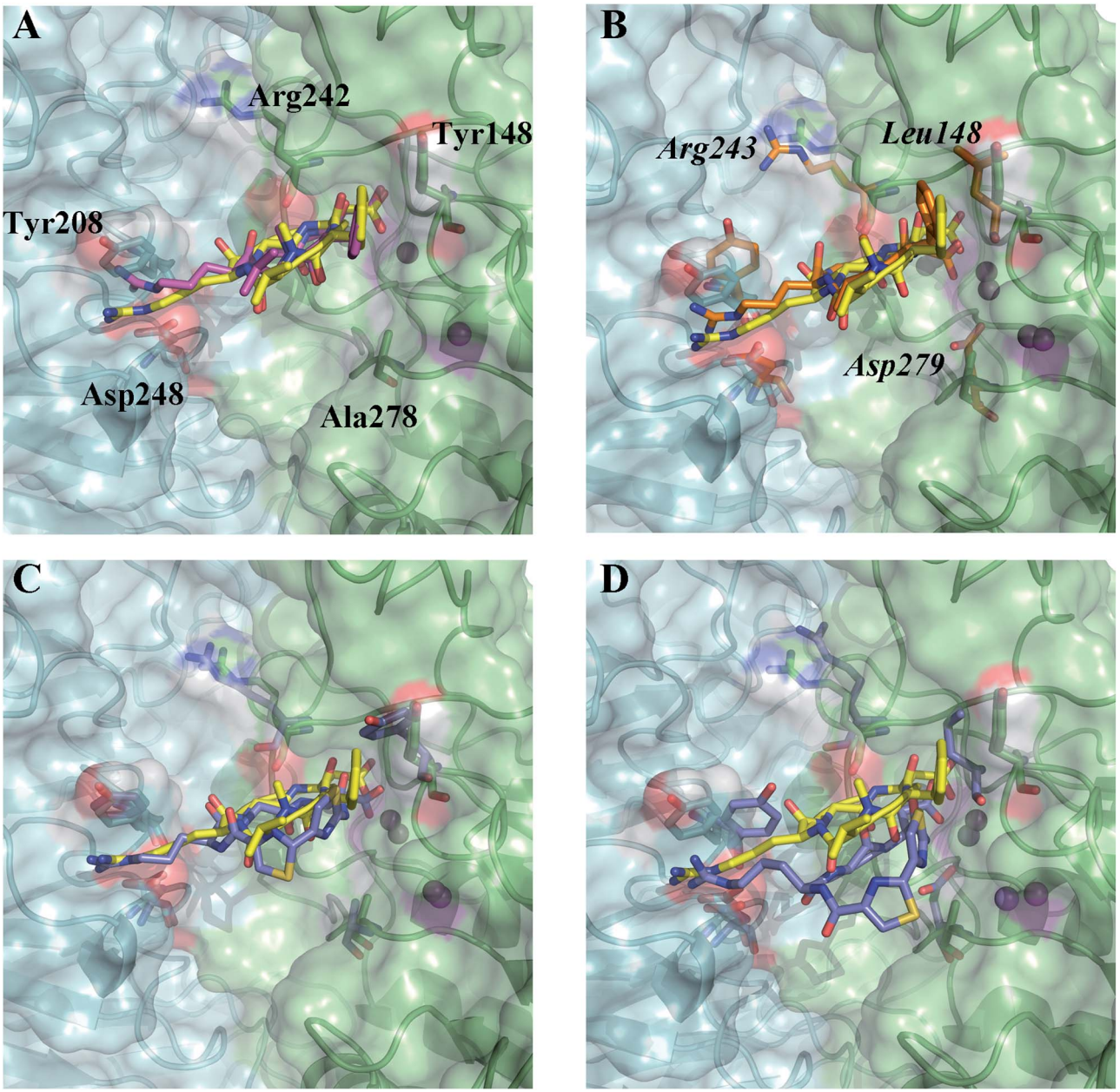


Fig. 3 (A) Representation of cilengitide bound to $\alpha_v\beta_3$ in the X-ray structure (PDB entry: 1L5G; magenta sticks) and in the last snapshot of the MD trajectory (yellow sticks). The α_v and β_3 units are shown as cyan and green surfaces, and selected residues are shown as sticks. (B) Representation of cilengitide in the last snapshots of the MD trajectories run for $\alpha_v\beta_3$ (yellow sticks) and $\alpha_v\beta_5$ (orange sticks). Selected residues in $\alpha_v\beta_5$ are shown as orange sticks (labelled in italics). (C) Superposition of cilengitide (yellow sticks) and compound **2** (blue sticks) bound to the last snapshots of the MD trajectories run for $\alpha_v\beta_3$. (D) Superposition of cilengitide (yellow sticks) and compound **2** (blue sticks) bound to the last snapshots of the MD trajectories run for $\alpha_v\beta_3$ and $\alpha_v\beta_5$, respectively. In all cases the metal cations are shown as magenta spheres. Residues are numbered according to the sequences in Uniprot ID P06756 (α_v), P05106 (β_3) and P18084 (β_5).

Table 2 Energetic contributions (kcal mol^{-1}) to the interaction of cilengitide and compound **2** and their predicted binding affinities

	Integrin	van der Waals	Electrostatic	Binding affinity
A	$\alpha_v\beta_3$	−29.1	−29.3	−10.8
	$\alpha_v\beta_5$	−31.4	−24.0	−11.5
2	$\alpha_v\beta_3$	−24.5	−26.4	−8.1
	$\alpha_v\beta_5$	−24.2	−22.2	−6.1

(Fig. 3C and D). This structural rearrangement can be attributed to the net effect arising from the electrostatic influence of Asp279, which points toward the NH groups proximal to the thiazole ring and the Asp residue, and the lack of stabilizing van der Waals contacts with Leu148, leading in turn to the loss of the cation–π interaction formed by the Arg residue with Tyr208, and of the hydrogen bond formed by the Asp NH unit with Arg243.

The similar structural arrangement of **A** in $\alpha_v\beta_3$ and $\alpha_v\beta_5$ was reflected in the similar van der Waals component of the ligand–receptor interaction (−29.1 *vs.* −31.4, respectively; Table 2), whereas the electrostatic component was less favourable in $\alpha_v\beta_5$, which reflects the electrostatic repulsion between the Asp residue in **A** and Asp279 (replaced by Ala278 in β_3). Overall, MM/PBSA calculations predict similar affinities for the binding of cilengitide to both $\alpha_v\beta_3$ and $\alpha_v\beta_5$ (Table 2), which is in agreement with the similar experimental activities obtained from the DAOY assays (Table 1). As expected from the loss of contact formed by the Phe residue in cilengitide (replaced by a thiazole ring in **2**), the van der Waals interaction energy was less favorable for the binding of **2**. However, the electrostatic term favored the interaction with $\alpha_v\beta_3$ by ∼4 kcal mol^{-1}. As a result, binding of **2** to $\alpha_v\beta_3$ was predicted to be more favorable than to $\alpha_v\beta_5$, which agrees qualitatively with the ∼70-fold increase in selectivity determined experimentally for compound **2** (Table 1).

Conclusions

In summary, a modular strategy for the preparation of a new (poly)thiazole moiety containing cyclic peptides has been described. As the key derivative is an amino acid that is prepared by an iterative protocol based on the Hantzsch thiazole reaction, analogues can be prepared, and therefore size, length and structural issues can be suitably modulated. This approach has allowed the preparation of cyclopeptide **2** with a similar potency and considerably increased integrin selectivity compared to the parent cilengitide. From a molecular point of view, it is likely that cyclopeptide **1** was overstrained or distorted and thus failed to properly display the recognition sequence for the RGD receptor cleft.

On the other hand, compounds **2** and **3** showed a satisfactory level of receptor occupancy, the former probably being better suited for binding with the SDL in integrin $\alpha_v\beta_3$ than with the $\alpha_v\beta_5$ protein (discriminating more efficiently than cilengitide). This fact is particularly relevant, since $\alpha_v\beta_3$ integrin is highly expressed in several tumor tissues, whereas $\alpha_v\beta_5$ is commonly found in healthy cells. These results open up new avenues for the design of and synthetic access to new analogues with enhanced integrin selectivity, a key issue for the development of theragnostic antiangiogenic agents.[23] We envisage that the "functional rigid connector" concept, exemplified here with the bisthiazole motif, will also have a broad range of applications for the synthesis of more rigid and drug like cyclic peptides with improved stability and selectivity.

Acknowledgements

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